MESOPOROUS SILICA NANOPARTICLES AS A BREAST CANCER TARGETING CONTRAST AGENT FOR ULTRASOUND IMAGING

by

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B.S., Tufts University, 2011

A thesis submitted to the
University of Colorado Denver
in partial fulfillment
of the requirements for the degree of
Master of Science
Bioengineering
2012
This thesis for the Master of Science
Degree by
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Bolin Liu

November 13th, 2012
Current clinical use of ultrasound for breast cancer diagnostics is strictly limited to a role as a supplementary detection method to other modalities, such as mammography or MRI. A major reason for ultrasound’s role as a secondary method is its inability to discern between cancerous and non-cancerous bodies of similar density, like dense calcifications or benign fibroadenomas. Its detection capabilities are further diminished by the variable density of the surrounding breast tissue with the progression of age.

Preliminary studies suggest that mesoporous silica nanoparticles (MSNs) are a good candidate as an in situ contrast agent for ultrasound. By tagging the silica particle surface with the cancer-targeting antibody trastuzumab (Herceptin), suspect regions of interest can be better identified in real time with standard ultrasound equipment. Once the silica-antibody conjugate is injected into the bloodstream and enters the cancerous growth’s vasculature, the antibody arm will bind to HER2, a cell surface receptor known to be dysfunctional or overexpressed in certain types of breast cancer. As more particles aggregate at the cell surface, backscatter of the ultrasonic waves increases as a result of the higher porous silica concentration. This translates to an increased contrast around the lesion boundary. Tumor detection through ultrasound contrast enhancement provides a tremendous advantage over current cancer diagnostics because it is significantly cheaper and can be monitored in real time.

Characterization of MCM-41 type MSNs suggests that these particles have sufficient stability and particle size distribution to penetrate through fenestrated tumor vasculature and accumulate in HER2+ breast cancer cells through the enhanced permeation and retention (EPR) effect. A study of acoustic properties showed that particle concentration is linearly correlated to image contrast in clinical frequency-range ultrasound, although less pronounced than typical microbubble-type contrast agents. In vitro studies using cells with varied levels of HER2 expression demonstrated the selectivity of the MSN-Herceptin conjugate to cells with HER2 overexpression. Fluorescence imaging suggest these images remain surface-bound and are not incorporated into the cell body.
This study demonstrates the potential of MSNs as a stable, safe, and effective imaging contrast agent for ultrasound-based cancer diagnostics. Ultimately this work will contribute towards the improvement of diagnostic alternatives to conventional ionizing radiation-intensive imaging – such as MRI or X-ray – without compromising the specificity of the test.

The form and content of this abstract are approved. I recommend its publication.

Approved by Robin Shandas
ACKNOWLEDGEMENTS

Foremost, I would like to thank my committee members – Dr. Daewon Park, Dr. Bolin Liu, and Dr. Robin Shandas – for taking the time over the past year to provide me with guidance throughout the project. I would also like to thank Luciano Mazzaro for his assistance with both ultrasound equipment and contrast agents, and Dr. Qun Li for her assistance with the cellular assays. Dr. Dunghwa Yun was instrumental in assisting with chemistry-related advice and solutions. Dr. Lara Hardesty and Dr. Ann Scherzinger (U. of Colorado Hospital) provided images and knowledge of clinical breast cancer diagnostics. Figures from Martin E. Anderson (Duke University) were adapted for Faran modeling and speckle distribution.
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1. Introduction

1.1 Review of breast cancer ultrasound diagnostics

Ultrasound used for breast cancer detection can accurately determine if a mass is a benign fluid-filled cyst, or a potentially malignant solid mass. A sonogram of a malignant tumor is characterized by:

- Lack of circumscribed margins
- Heterogeneous echo patterns
- Increased anteroposterior dimension (‘taller than wide’)

Unfortunately, it is very difficult to determine if a detected solid mass is truly a malignant carcinoma, or simply a calcification or fibroadenoma since all of these cases yield similar densities. Malignant tumors are typically hypoechoic, but may either be hyperechoic or isoechoic as well (Figure 1.A). The detection of the mass diameter may also be difficult to determine if the surrounding tissue is denser than normal breast tissue, which is common in women below the age of 50. In the clinical setting, ultrasound is typically administered in conjunction with another standard method of diagnosis, most commonly mammography. One study found that ultrasound adjunct to mammography was able to increase the incremental rate of detection by up to 41% [1]. Remarkably, ultrasound actually has a higher specificity than mammography, is less affected by surrounding tissue density as a result of age, and can detect smaller diameter lesions [2]. However, its inability to stand alone as a primary method of detection is mostly due to a high false positive rate [3]. Surgical biopsies of lumps that showed a positive ultrasound following a negative mammogram were determined to be benign in 3% of the cases [4]. Depending on the age group, adding ultrasound to mammography may quadruple the false positive rate. To further improve detection rates following diagnostic ultrasound, biopsies are often included. With an average cost around $500, biopsies require time from both the sonographer and the pathologist. It may also take up to a few days to return the results. Due to the discomfort of the large bore needles, local anesthetics must also be used. Some patients have expressed concern with the possibility of contaminating new regions from cancer cells on the needle as it is pulled out of the tissue.
Ultrasound is also commonly used for needle guidance for biopsy as well as localization for surgical removal. If a lesion has been determined to be cancerous, it is marked for removal prior to surgery by placing a wire across the diameter of the cancerous region (Figure 1.B). The accuracy of this method of occult lesion localization is dependent upon the sonographer’s ability to distinguish the tumor boundary edges. Again, local anesthetics must be used to accommodate for the large bore needle. During surgery, the wire is located either visually or with intraoperative ultrasound. A recent study suggests that around 33% of oncological surgeons use intraoperative ultrasound regularly\(^5\). With or without ultrasound, the extent of tissue removed around the wire is determined by palpation. Since surgeons must rely on a one-dimensional marker, excess tissue must be removed as a cautionary measure (Figure 1.C).

![Figure 1. Ultrasound imaging of cancerous breast tissue. (A) Typical indication of malignant tumor, (B) Needle inserted through suspected tumor for localization wire injection, (C) MRI of excised tissue with localization wire and biopsy site clip injected during US imaging](image)

It is evident that ultrasound holds many advantages over current standards of diagnosis. Once diagnosis specificity is improved upon, ultrasound-based diagnostics may prove to be adequate as a primary method of detection comparable to mammography. Diagnostic ultrasound for breast cancer and other HER2 positive cancers can benefit from contrast enhancement for detection and presurgical occult lesion localization

### 1.2 Review of Herceptin

Herceptin is the trade-name given by Genentech\(^\text{™}\) for the cancer treatment therapy trastuzumab. At 155 kDa, trastuzumab is a humanized monoclonal antibody that has been developed to interfere with the functionality of the HER2/neu or ErbB2 receptor. When a secondary receptor such as HER3 binds to an external stimulus, it dimerizes with a HER2/neu receptor. The activated HER2/neu receptor in turn initiates a MAPK and PI3/AKT kinase
pathway, subsequently initiating a NF-kB growth pathway\[6\]. By this chain of events, an external signal can regulate cellular proliferation, migration, differentiation, and adhesion. Certain types of cancers – particularly types of breast cancer – have been shown to overexpress the HER2/neu receptor by 25% up to 100 fold\[7\]. Uncontrolled growth as a result of overexpressed HER2/neu receptors occurs by two mechanisms: (1) A high concentration of HER2/neu receptors on the cell surface (>2 million/cell) leads to a tendency for hyperactivity, initiating cell growth pathways without external mitogen stimulation, or (2) The constitutive activation of the AKT pathway interrupts the p27Kip1 checkpoint\[8\]. Since p27Kip1 is constantly in a phosphorylated state, it begins to accumulate in the cytoplasm, unable to reach the nucleus. A loss of inhibition to the cdk2 pathway by p27Kip1 results in an exacerbated effect from the HER2/neu overexpression.

Trastuzumab interferes with HER2/neu hyperactivity by disrupting the dimerization step prior to pathway initiation. To date, it is FDA approved for treatment of certain breast cancers, as well as stomach, gastroesophageal junction, and uterine papillary cancers\[9\]. Prior to treatment, the region of interest must be screened to confirm that the specific type of cancer exhibits overexpression of HER2/neu receptors on the surface. Immunohistochemistry or fluorescence in situ hybridization (FISH) of a biopsy section can sufficiently determine the quantity of HER2/neu receptors on the cell surface. Despite a high binding affinity of trastuzumab to the HER2/neu receptor, a recent study has shown that nearly 70% of patients undergoing treatment with trastuzumab alone do not respond to the treatment\[10\]. This abysmal statistic may be explained by patient-to-patient variation in resistance. The resistance may arise as a de novo primary resistance, or become acquired in a longterm setting. Herein presents an opportunity to improve trastuzumab as a cancer treatment option. Currently, trastuzumab is only applied as an adjuvant treatment, either administered concurrently with chemotherapeutics or post-surgery. One study found that trastuzumab treatment post-surgery can reduce relapse in patients by 50\%\[11\].

Since trastuzumab is so highly selective for HER2 receptors, the drug has recently become a favorable agent for cell targeting. Liu et al. were able to successfully conjugate the trastuzumab antibody to a liposomal drug delivery vehicle for a camptothecin anticancer drug. The results showed that immunotargeting with trastuzumab improved cellular uptake from overexpressing HER2 cancer cells by 50-300 fold\[12\]. Prior studies have also shown that Anti-HER2 antibodies can maintain their anti-tumor efficacy despite conjugation to another body\[13\].
1.3 Review of ultrasound contrast

Ultrasound waves are longitudinal compression waves caused by pulses of pressure propagating in a direction away from the source. These pulses create a compression and expansion (rarefaction) effect that causes ripples to propagate through a medium. Waves are able to travel through media since most media have some degree of elasticity. These waves are considered ultrasonic since its frequency is above the audible spectrum of the human ear (~20 kHz).

Depending on the medium, a certain amount of the ultrasound wave returns back in the direction of the source. The amount of energy returned to the transducer (source) is used to calculate the elasticity and density of a medium at different depths. By creating an array of transmitting/receiving transducers, a 2D image can be created. The amount of energy returned to the transducer is calculated as the pixel intensity at a certain depth. White pixels correspond to more energy returned to the transducer, whereas black pixels correspond to less energy returned to the transducer. The extent of returned energy from the initial wave pulse is dictated by several mechanisms.

1.3.1 Reflection by Rayleigh-like scattering

Most physiologically relevant media exhibit some form of inhomogeneity. On ultrasound images, the result is a snowy appearance defined as ‘speckling’. The speckle pattern of each tissue is unique. While some groups are currently investigating the possibility of calculating speckle pattern to determine tissue composition, the observation of speckle pattern in a clinical setting is still primarily qualitative.

Speckle patterns are caused by inhomogeneities whose diameter is less than the axial resolution of the imaging system. The interaction of these sub-resolution particles and an incident sound wave may be defined as Rayleigh-like scatters. First used to describe particle interactions with light, Rayleigh scattering principles may also be applied to describe the interaction of sound waves with particles by substituting several parameters. Any inhomogeneity whose density or compressibility is different from the surrounding medium can be defined as ‘point scatterer’. The interaction of sound waves at an interface of differing density or compressibility acts as a source for a secondary sound wave. This secondary wave propagates in a spherical manner concentric to the point scatterer, as described by Huygen’s principal (also originating in the context of light waves)\cite{14}. The magnitude of the secondary
wave in the direction of the initial source defines the backscatter. According to the Faran model\cite{15}, the amplitude of the backscatter is primarily dependent on its $ka$ value, or its wavenumber multiplied by the point scatterer’s radius. Under Faran’s model of a solid sphere introduced to planar orthogonal pressure wave, the echo intensity follows a 4th order dependence when $ka$ is less than 1 – similar to Rayleigh scatterers in the context of light waves (Figure 2, left). As the radius increases, the echo amplitude drastically increases as well. Assuming that the incident sound wave remains constant ($k$), it is clear that the echo intensity will also exponentially decreases as the particle radius decreases (Figure 1, left). Neglecting other phenomena (such as harmonic oscillation or attenuation), a 1µm diameter particle observed by a 10 MHz wave in water ($c = 1493 m/s$) yields an insignificant echo response ($ka = 2\pi/\lambda = 5e^{-7*2\pi/3e^{-4}} = 0.02$). As such, contrast agents must take advantage of other phenomena in order to effectively create backscatter.

![Figure 2](image)

**Figure 2.** Backscatter intensity as a function of wave frequency ($k$) and particle diameter ($a$)

The Faran model also reveals a strong frequency dependence for echo intensity (Figure 2, right). When the sub-resolution particle diameter remains constant, echo intensity (dashed line) displays strong nodes and peaks as a function of frequency (proportional to wavenumber, $k$). As frequency increases, resolution improves echo intensity becomes less frequency dependent.

### 1.3.2 Reflection by variation at media boundaries

Most relevant ultrasound contrast is introduced at the interface between two media on a macroscale (>1mm). This is primarily due to the reduction of acoustic mismatch between the contrast agent and the surround tissue. Image contrast intensity is largely governed by the
acoustic mismatch, or difference between acoustic impedance at an interface of two difference media. The acoustic impedance \( Z \) can be defined as:

\[
Z = \rho c; \quad c = \frac{B}{\sqrt{\rho}}
\]  

(1)

The acoustic wave velocity, \( c \), is the quotient of a medium's density and its bulk modulus, \( B \). In gasses, the bulk modulus is calculated as the product of the gas' inherent adiabatic index (the coefficient that correlates a change in volume to its responsive change in temperature) and the ambient pressure. The bulk modulus of a solid is typically determined experimentally.

By knowing the acoustic impedance of two media, the contrast induced at the boundary between these two media can be theoretically calculated by the intensity reflection coefficient equation:

\[
R_i = \left( \frac{Z_2 - Z_1}{Z_2 + Z_1} \right)^2
\]

(2)

Where \( Z_1 \) defines the media through which the incident sound wave initially interacts, and \( Z_2 \) defines the secondary media of interaction. This form of the equation assumes that the incident sound wave is perpendicular to the boundary surface. Angle approaches are considered using Snell’s Law:

\[
R_i = \left( \frac{(Z_2/c_2\cos\theta_1 - Z_1/c_1\cos\theta_1)^2}{(Z_2/c_2\cos\theta_1 + Z_1/c_1\cos\theta_1)^2} \right)
\]

where \( \frac{\sin\theta_i}{\sin\theta_t} = \frac{\lambda_1}{\lambda_2} = \frac{c_1}{c_2} \)

(3)

Figure 3. Incident wave through two media at angle theta

By substituting equation (1) into the \( Z \) variables of equation (2), it is apparent that the intensity reflection coefficient is solely determined by differences in density and stiffness (bulk modulus).
By having a low density and a low acoustic wave velocity (as a result of a small bulk modulus),
gasses provide an excellent acoustic impedance mismatch to soft tissue. Alternatively, by having
a density and acoustic wave velocity higher than soft tissue, solid materials can also yield strong
intensity reflections at a soft tissue boundary.

1.3.3 Reflection by attenuation

The majority of ultrasound imaging is dependent upon backscatter (‘echo’) caused by
boundary surfaces of different density or compressibility. By this mechanism, the contrast
intensity is determined by how much wave energy is reflected back to the transducer, and how
much energy is either transmitted across the boundary or scattered in a direction away from the
transducer. Ultimately, contrast is determined by the difference between the magnitude of
wave energy received and the original pulse wave energy. Another mechanism of energy loss is
caused by converting acoustic wave energy into another form, such as heat. The result on a
sonogram would be a darker region, defined as ‘anechoic’. This loss in energy also affects
regions beneath the anechoic region, resulting in a phenomenon known as shadowing (Figure
4).

![Ultrasound image of breast displaying shadowing caused by attenuation. Image obtained from http://nexradiology.blogspot.com](http://nexradiology.blogspot.com)

Attenuation – the conversion of acoustic energy to another form – can be quantified by the
following equation:

\[ H(f, z) = a_0 f z \]  

(4)
The degree of attenuation is dependent upon the depth of the region of interest, \( z \), as well as the sound wave frequency, \( f \). As depth and frequency increases, the attenuation of the initial pulse also increases. The attenuation coefficient, \( a_o \), is a constant value inherent to the medium commonly expressed in terms of dB/(cm MHz).

Attenuation can be caused by small particles whose diameter is much smaller than the sound wavelength. In 1972 J.R. Allegra and S.A. Hawley published a landmark paper defining the parameters that affect attenuation in suspensions and emulsion [16]. Using 0.44-0.653 \( \mu \)m diameter polystyrene spheres suspended in water or toluene introduced to sound waves across a 9-165 MHz range, Allegra and Hawley were able to accurately predict the attenuation of the initial sound wave in each experiment. The proposed model applies six boundary conditions through six differential equations. The mathematics of these equations is beyond the scope of this thesis, but several concepts are taken into consideration:

- The incident sound wave can be characterized by three wave equations (compressional, shear, and thermal);
- Linearized conservation laws apply to the system. Denser objects with more inertia will have less spatial displacement from compressional waves. Since denser solid particles will move less in relation to the surrounding fluid (or soft tissue), flow is observed across the particle surface. This flow introduces viscous drag. This viscous flow justifies the addition of a shear wave component to describe the incident sound wave. As viscosity increases, heat created from friction increases and acoustic wave energy is lost.
- Thermal conduction can be considered, according to thermodynamic laws of pressure-temperature coupling. Depending of the specific heat of a material, thermal loss can be related to pressure increases from ultrasonic wave pulses.

1.4 Ultrasound Enhanced Contrast Agent (UECA)

A vast majority of FDA approved contrast agents are gas phase microbubbles that are encapsulated in either liposomal or protein carriers. Table 1 provides a summary of the three most widely used contrast agents for ultrasound imaging.
Table 1. Summary of current contrast agents

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Sonovue</th>
<th>Optison</th>
<th>Definity</th>
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<tbody>
<tr>
<td>Gas</td>
<td>Sulphur hexafluoride</td>
<td>Perfluoropropane</td>
<td>Perfluoropropane</td>
</tr>
<tr>
<td>Mean bubble size</td>
<td>2-8 µm</td>
<td>3.0-4.5 µm</td>
<td>1.1-2.5 µm</td>
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<tr>
<td>Shell composition</td>
<td>Phospholipid</td>
<td>Human albumin</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>Manufacturer</td>
<td>Bracco</td>
<td>GE healthcare</td>
<td>Bristol Myers Squibb</td>
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All three contrast agents are applied as an agent to increase the contrast of blood flow through dynamics systems, particularly through the heart. Figure 5 provides an illustration of the enhanced contrast of blood when injected with a 1 mL bolus of Sonovue®.

Figure 5. Comparison of ventricular filling (A) before and (B) after Sonovue injection

This class of contrast agents is “indicated for use in patients with suboptimal echocardiograms to opacify the left ventricular chamber and to improve the delineation of the left ventricular endocardial border”[17].

Figure 6. Chemical structure of Perfluoropropane
All three contrast agents employ some form of heavy gasses (Figure 6). Unlike lighter gasses (including air), these gasses have a lower solubility which consequentially increases the half-life in the bloodstream. Regardless, the half-life of these bubbles is restricted to several hours. Due to the relatively large diameter, most bubbles are cleared from the system much sooner. Since all currently approved microbubbles are larger than 1µm, these contrast agents are unable to pass through endothelial barriers. 96% of perfluoropropane gas in a 20mL bolus of Optison™ is eliminated through the lungs within 10 minutes [18]. The albumin shell is most likely eliminated by liver degradation. Heavy gasses such as perfluoropropane provide the contrast effect typical of gas to solid/liquid phase acoustic mismatch (Section 1.3.2, ‘Reflection by variation at media boundaries’). Another mechanism by which contrast is increased is through harmonic oscillations created by ultrasound waves close to the bubble resonant frequency [19]. This contrast effect is caused in part by the elasticity of the bubble’s geometry. Unfortunately, this high degree of elasticity also contributes to its susceptibility to rupture. As a result, imaging time under continuous wave pressure is limited to the order of minutes.

Several groups have previously attempted to apply MSNs as a UECA [20]. Casciaro et al. and Liu et al. successfully correlated mean pixel intensity of an image to the concentration of 330-660nm diameter MSNs within a clinically relevant frequency range. In vivo studies suggested that untargeted MSNs aggregate within Browicz-Kupffer cells as a part of the reticuloendothelial system (mononuclear phagocyte system) in the liver. The aggregate concentration within the liver is significant enough to be detected by radio frequency-range ultrasound. At the time of this publication, no groups have reported findings on targeted MSN contrast agent effects. Furthermore, no groups have provided an in-depth computational justification for MSN usage as a contrast agent.

1.5 Mesoporous silica nanoparticles

1.5.1 Composition

Mesoporous silica is most similar to synthetic zeolites, a class of silicate mineral that is notable for its porous nature. The porosity of zeolite has made the mineral ideal for commercial applications such as absorbents and purification filters. The “mesoporous” descriptor of the silica nanoparticles is defined by the International Union of Pure and Applied Chemistry (IUPAC) classification of porous materials. Any inorganic structure whose pore diameter is between 20 Å and 500 Å is classified as mesoporous. Anything above 500 Å would be macroporous, whereas
anything below 20 Å would be microporous. Although several various microstructures exist, most follow the general method exemplified from the production of Mobil Composition of Matter (MCM)-41 (Figure 7). For MCM-41, the surfactant cetyltrimethylammonium bromide (CTAB) naturally creates liquid crystalline micelles in water. This will act as the template for the pore structure. The ceramic sol-gel precursor tetraethylorthosilicate (TEOS) creates a silica network around the micellar formations through hydrolysis and condensation. Once the CTAB template is extracted through solvent extraction or calcination (thermal treatment), a hexagonally ordered silica framework remains.

Compared to other mesoporous silicas, MCM-41 has a large surface area (941 m²/g) and large pore volume (0.73 mL/g). Up to 80% of its volume can be pore space. The walls of its hexagonal structure is primarily composed of amorphous silica (SiO₂), making it less mechanically stable than other more crystalline zeolites.

1.5.2 Biomedical applications of MSNs

**Drug Delivery Vehicle:** Since 2001, MSNs have been gaining momentum as a platform for drug delivery systems. Originally designed as molecular sieves, these particles have several advantageous characteristics, including:

- Large surface area for functionalization and interaction with the environment
- Large pore volume for drug loading
- Highly ordered pore network for control of loading and release kinetics
- Silanol-containing surface for easy functionalization
- Low immunogenicity, with an ability to naturally be incorporated into the cell by endocytosis

MSNs as a drug delivery system has been applied to several indications to act as a low release or site targeting drug reservoir. Recently, MSNs have been applied as an implantable drug delivery system for bone tissue, thanks to its favorable properties as a bioceramic[21]. MSNs have also been used as a form of biosensors by loading pore space with bioactive dyes, which only fluoresce under the presence of specific stimuli[22]. The functionalization of the pore interiors allow hydrophobic drugs with low solubility to be delivered to target sites more easily. To avoid premature release of the loaded drug, methods for capping pores has become a research area with growing interest. Capping using soft material (antibodies, insulin, pH/temperature sensitive polymers) or hard materials (gold, cadmium sulfide, iron oxide) provides tight control the drug delivery package. Cap release can be achieved by both external stimuli (magnetic fields) as well as internal stimuli (glucose, pH, temperature).

In a previous study, Liong et al. had previously functionalized the surface of MSN particles by conjugating the surface with folic acid[23]. Alpha-folate receptors have been shown to be upregulated in certain human cancers. Liong was able to show the preferential absorption of the MSN into the cancer cell lines PANC-1 and BxPC3. Furthermore, the MSNs incorporated into the cancerous cells were able to release their camptothecin drug payload into the cytosol, effectively reducing cell survival by 60%. Evidently, MSNs are an efficient platform for cancer drug delivery systems.
2. Aims and experimental approach

2.1 Specific Aims

2.1.1 Demonstrate the contrast potential of mesoporous silica nanoparticles

Silica as a contrast medium offers several advantages over microbubbles. Above all, silica offers a multifunctional platform that is easier to modify than its microbubble counterpart. In order to apply silica nanoparticles as a contrast agent, the particles must demonstrate the ability to significantly alter the contrast of a region of interest via conventional ultrasound.

Experimental approach: In order to demonstrate the physical effect of ultrasonic waves on MSNs, the system was initially modeled as a colloidal solid particle distributed throughout a homogenous phase acoustically similar to soft tissue. The interaction of ultrasonic pressure waves throughout the system could then be characterized using an Ingaard-Morse model for acoustic scatterers. By using the Ingaard-Morse model to define thermodynamic and hydrodynamic interaction between the particle and phase under ultrasonic pressure waves, a sufficient prediction could be made for the contrast effects during imaging. These predictions were confirmed by B-mode ultrasound image analysis of agar or acrylamide blocks with varying concentrations and diameters of silica nanoparticles.

2.1.2 Apply MSN as an ultrasound contrast agent through cancer cell targeting

A major reason ultrasound is not more widely used for breast cancer diagnostics is its inability to discern between cancerous and non-cancerous masses of the same density. If the contrast of a cancerous mass can be enhanced in real time, ultrasound will be a far more effective means of cancer diagnostics. In order to use MSNs as a contrast agent for breast cancer, the particles must be selective as to where they accumulate. The particle’s functionality is primarily achieved by three mechanisms:

1. Size: by having a diameter less than 1µm, MSNs are able to pass through the fenestrated endothelial walls of tumor vasculature.

2. HER2 receptor targeting: prior research has shown that certain types of breast cancer overexpress the HER2 surface receptor by 100 fold\(^{24}\). This overexpression makes the HER2 surface receptor an ideal targeting antigen for cancerous cells. Herceptin, an anti-HER2 antibody, was conjugated to the surface of MSNs to ensure accumulation near tumor regions.
Porosity: by maintaining a porous interior structure, variations in density and compressibility will enhance the reflectivity of incident soundwaves, further increasing echo contrast during imaging. By adjusting the size and surface of the MSN, the local volume fraction of MSNs in and around the tumorous tissue will increase and only the targeted regions of interest will experience an enhancement in contrast.

**Experimental approach:** To ensure that both size and surface modification were optimized for accumulation in vivo, particle size distribution (PSD) and cellular interaction was first characterized. PSD was analyzed by scanning electron microscope (SEM) and tunneling electron microscope (TEM) imaging. Surface modification steps were monitored by Fourier transform infrared spectrometry (FTIR), SEM, and ultraviolet-visible spectroscopy (UV-VIS). To observe using fluorescence microscopy and fluorescence-activated cell sorting (FACS), the MSN-Herceptin conjugates were labeled with fluorescein isothiocyanate (FITC). The FITC-labeled MSN-Herceptin was introduced to four different cell lines of increasing expression of the HER2 receptor.

### 2.2 Experimental Obstacles

#### 2.2.1 Particle selection

In order to be an effective targeting agent for passage through fenestrated tumor vasculature and target cell attachment, MSNs must fall within a specific diameter range. After extensive characterization, it is clear that the commercially available MCM-41 yielded a wide particle size distribution (PSD) with a mean diameter far greater than 8µm. Since MSNs essentially comprise of stacked hexagonal pore sheets, the mean diameter could be modestly reduced by sonication, but could not disintegrate fully intact sheets. Thus, the bulk material must be sieved to collect the particles population with desired diameter.

#### 2.2.2 Sieving

Due to its size, surface charge, and density, MSNs are incapable of dissolving in solution. Furthermore, these colloidal particles do not remain uniformly suspended in solution longer than an order of minutes. In general, the issue of insolubility was accommodated by sonication and use of ethanol as a solvent (more hydrogen bonding with silanol or amine groups). However, colloid flocculation posed a consistent problem in terms of filtration. Once it was
determined that the initially proposed Sepharose® column could not accommodate the larger silica particles, a simpler paper-based 1µm filter was applied. Unfortunately, less than 1% yield was obtained after filtration. One suggested explanation for this loss of product was channel clogging caused by larger diameter particle. Finally, a 1-2 layer woven nylon grid mesh with 1 or 8µm pore diameters was applied. Since pore channels were shorter (75µm) and less circuitous, the upstream flocculation layer could be more easily disrupted by retrograde flow or sonication. Regardless, the filtered yield rarely exceeded 40% of the initial mass.
3. Materials and methods

3.1 Materials

3.1.1 Consumables

MCM-41 type (hexagonal) MSNs were purchased through Sigma-Aldrich (Milwaukee, WI) and filtered through a 1µm nylon mesh (Elko Filtration Inc., Miami, Fl). 1N hydrochloric acid, ethanol (200proof), anhydrous toluene, 1X Phosphate buffered saline (PBS), (3-aminopropyl)triethoxysilane (APTES), dimethylsulfoxide (DMSO), and fluorescein isothiocyanate isomer I (FITC) for MSN functionalization were provided by Sigma-Aldrich (Milwaukee, WI). Trastuzumab (Herceptin®) was provided by Dr. Bolin Liu. For ultrasound phantoms, acrylamide, N,N’-methylene bis(acrylamide), ammonium persulfate (AmPs), and Tetramethylethylenediamine (TEMED) was used for solid tumor mass mimics.

For in vitro studies, two groups of increasing HER2 expression were used: (1) MDA-MB-231(TN) has a very low level HER2 expression, and (2) BT474 or SKBR3 yields a moderate level of HER2 expression. All cell lines were provided courtesy of Dr. Bolin Liu’s group (University of Colorado Department of Pathology). The MDA-MB-231(TN) cell line was cultured in RPMI-1640 with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PS). The cancer cell lines (BT474, SKBR3, and HR20) were cultured in Dulbecco’s Modified Eagle Medium (DMEM)/F12 with 10% FBS and 1% PS.

For transmission electron microscopy (TEM), samples were stained with 2% uranyl acetate and placed on 400 mesh formvar-coated, carbon evaporated, and glow discharged copper grids, courtesy of the University of Colorado –Denver Core facilities.

3.1.2 Equipment

To disrupt aggregation prior to use, all MSNs were sonicated in an ultrasonic waterbath cleaner operating at 35kHz for 10 minutes. All MSN particles were dried in a 100°C oven.

For characterization with TEM, samples were imaged on a Tecnai G2 series scope (FEI™, Hillsboro, OR). Low vacuum scanning electron microscopy (SEM) was performed on a JSM-6480LV W-thermal emission microscope (JEOL Ltd.™, Peabody, MA), at the University of Colorado-Boulder Nanomaterials Characterization Facilities (NCF).

Single-pulse ultrasound measurements were obtained using Non-Destructive Testing (NDT) transducers with matching frequencies ranging from 0.5-7.5 MHz (GE-Panametric™). All
transducers were Accuscan type S immersion transducers with point target focus (PTF) and 1” focus length under water. Two transducers were set facing each other through the round window of a 12” X 8” X 3” acrylic container (Figure 8). The box was filled with agar gel at the bottom (or an agar cylinder was placed inside) and filled with deionized water. The water was retained within the container by covering the window with double layered masking tape.

To ensure clean connectivity, ultrasound gel was applied between the single element transducers and the masking tape-covered window. The precise distance between transducers was measured prior to each set of experiments. Each transducer was designated as a pulser or receiver and attached to a Panametric NDT Model 5800 pulser/receiver. Receiver signal was acquired on an Infinium 8000 High Performance Oscilloscope (Agilent Technologies™). Waveform data was sent to MATLAB (The Mathworks, Inc. ™ Natick, MA) for data processing.

B-Mode imaging was acquired on a Sonix SP clinical ultrasound monitor attached to a L14-5/38 curvilinear probe (Ultrasonix, BC, Canada). All images were taken inside of the acrylic containterr. Images were processed in ImageJ (NIH, Bethesda, MD) and MATLAB (The Mathworks, Inc, Natick, MA).

Fluorescence-activated cell sorting (FACS) was performed on a FACScan (BD, Franklin Lakes, NJ) and processed through FlowJO™
3.2 Methods

3.2.1 Characterization of mesoporous silica nanoparticles

**MSN preparation:** Prefabricated MCM-41 hexagonal type MSNs were dispersed in 200 proof ethanol at a concentration of 5 mg mL\(^{-1}\) and bath sonicated for 10 minutes. The dispersion was then filtered through either a 1,8,10 and 30 µm nylon mesh from a 10 mL syringe. Retrograde/prograde flow alternation in a 1 sec : 3 sec timing pattern was used to prevent flocculation on the upstream side of the filter. The filtered particles were dried in a 100°C oven for 48 hours.

**TEM imaging:** Dried MSN was resuspended in 200 proof ethanol to 500 µg mL\(^{-1}\) and stained in 2% uranyl acetate for 3 minutes. After drying on a 400 mesh formvar coated copper grid, the samples were imaged at 80kV.

**SEM imaging:** Dried MSN was resuspended in 200 proof ethanol to 500 µg mL\(^{-1}\). 20 µL drops were placed onto glass slides, allowing for the ethanol to evaporate off. The glass slides were then sputter coated in a gold/platinum alloy to a 3 nm thickness. Images were acquired at 5.0 kV.

3.2.2 Determination of acoustics properties

**Single pulse scan:** To quantify the attenuation caused by MSNs, signal loss through an acrylamide block was measured. This method was also used to calibrate the acoustic properties of the acrylamide blocks to better represent soft tissue. Using the apparatus previously described (Section 3.1.2, “acoustic measurement apparatus”), acrylamide blocks with incrementally increasing concentrations of 1 µm MSNs (0, 0.1, 0.25, 0.5, 1, 2, 5, and 10 mg mL\(^{-1}\)) were placed into the acoustic measurement apparatus. The system apparatus was left untouched for 5 minutes to allow for all air bubbles and potential contaminants to settle. 25 µJ pulses were transmitted through the MSN/water samples at 5 kHz. The waveform was averaged and transferred to a MATLAB program for analysis (Appendix A). To calculate the attenuation, a fast fourier transform was performed and deconvolved with a reference signal of pure water, as described by Laugier et al. \(^{[25]}\). The complex spectrum of a signal can be described by the following equation:

\[
A(f) = A_0(f) e^{-2\pi f \left( \frac{1}{\nu_0} \right)}
\]  

(5)
Where $A_0(f)$ is the systems response, $f$ is the frequency, $L$ is the distance between the two transducers, and $v_o$ is the speed of sound through the medium. The frequency-dependent attenuation of the MSN-water dispersions can be derived from the ratio of its magnitude spectra to the magnitude spectra from a reference water signal’s complex signal, $A_r$:

$$\alpha(f)L = \ln \left( \frac{|A_r(f)|}{|A(f)|} \right)$$

\[ (6) \]

**B-mode imaging:** To calculate the correlation between image contrast and particle concentration, 25% polyacrylamide phantoms were created with incrementally increasing concentrations of 1 µm MSNs at 0, 0.1, 0.25, 0.5, 1, 2, 5, and 10 mg mL$^{-1}$. Acrylamide was selected due to its similarity to soft tissue’s acoustic properties \[26\]. The acrylamide phantoms were created by adding MSN to 25% acrylamide/5% N,N’-methylene bis(acrylamide). Prior to polymerization, the solution was sonicated for 3 minutes using a probe sonicator set to power level 3. After adding 0.05% AmPs and 0.025% TEMED, 1 mL was immediately poured into a 5 mL syringe with the nozzle removed. Complete polymerization occurred between 2-5 minutes. The polyacrylamide phantoms were submerged under water and placed on a 5% agar platform approximately 5 cm in height to separate the ultrasound probe’s far-field from the highly reflective bottom of the acoustic measurement apparatus. The phantoms were imaged with a 10 MHz probe at 70dB. Two focal points were set at the top and bottom of the phantom, with the top of the phantom 2.5 cm from the top of the phantom. 3 images were taken for each phantom and transferred to a MATLAB program for analysis (Appendix B). The interior of the phantom was parsed into 50 pixel x 50 pixel squares and measured for mean pixel intensity. Each square, along with the squares from the other two images of the same concentration, was then averaged. Each mean gray pixel intensity value was correlated to the MSN concentration.

### 3.2.3 Herceptin Conjugation to silica nanoparticles

**MSN functionalization:** Prefabricated MCM-41 hexagonal type MSNs were prepared as previously described (Section 3.2.1, “MSN preparation”). The 1µm diameter particles were resuspended in 10% HCl to a concentration of 31.4 mg mL$^{-1}$, in order to increase the amount of free silanol groups on the surface (Figure 9, step 1). After stirring for 1 hour at room temperature, the dispersion was centrifuged at 5,000 rpm for 5 minutes, decanted, and washed
in deionized water (\(\text{dH}_2\text{O}\)). This centrifugation and wash step was repeated two more times. After the third decantation, the particles were resuspended in \(\text{dH}_2\text{O}\) and sonicated for 10 minutes to reduce aggregation. The particles were dried in a 100°C oven for 48 hours. Silanol group concentration was predicted by FTIR and pH curve.

For amine functionalization, the hydroxylated MSNs were suspended in anhydrous toluene to a concentration of 20.7 mg mL\(^{-1}\). APTES was added to bring the final concentration to 6.5% (Figure 9, step 2). The dispersion was placed in a nitrogen environment by injecting a 1mL syringe with an 18G needle through the stopper on a round-bottom flask. The plunger of the syringe was replaced with a double-layered nitrogen-filled balloon (~10 cm diameter). The lip of the balloon was secured with parafilm. The dispersion was centrifuged at 5,000 rpm for 5 minutes, decanted, and washed in anhydrous toluene. To purify the particles from unbound APTES, the dispersion was added dropwise into ether, and again centrifuged 5,000 rpm for 5 minutes. This centrifugation and wash step was repeated two more times. After the third decantation, the particles were resuspended in 200 proof ethanol and sonicated for 10 minutes to reduce aggregation. The particles were dried in a 100°C oven for 48 hours.

**Herceptin/FITC conjugation:** To couple the Herceptin antibody with MSNs, amine groups on the silica surface were coupled to carboxylates from exposed aspartic and glutamic acid residues and carboxyl termini on the Herceptin antibody (Figure 9, step 3). After preparing a solution of 0.5mg/mL Herceptin in 1X PBS, N-hydroxyl succinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC) was added as a crosslinking agent (2mM final concentration for each). Amine-coupled MSNs were resuspended in the solution (4 mg/mL) and sonicated for 10 minutes to ensure optimal exposure to surface area. The EDC crosslinking reacted for 4 hours, stirring at room temperature. The product was then centrifuged for 10 minutes at 3,000 rpm and decanted to remove the water-soluble carbodiimide and urea byproduct. The product was resuspended in 1X PBS. The centrifugation, decantation, and resuspension step was repeated three times.
Figure 6. Chemical reaction of MSN-antibody linkage
Conjugation of modified MSN and Herceptin

Figure 7, continued. Chemical reaction of MSN-antibody linkage
After the third resuspension to 5 mg mL\(^{-1}\) in PBS, protein concentration was determined by Bradford Assay. Once the protein concentration was calculated, 10 mg mL\(^{-1}\) FITC in DMSO was introduced to a ratio of 80 µg FITC: 1mg Herceptin. 1 hour of reaction time stirring at room temperature in the dark allowed for FITC conjugation to uncoupled amines and sulfhydryl groups. The solution was dialyzed in 3,500 kDa molecular weight cutoff (MWCO) dialysis tubing in milliQ water. The water was changed 5 times over a period of 72 hours. Dialysis occurred at 4°C to preserve protein integrity. FITC conjugation was confirmed by fluorescence microscopy and UV/VIS. FITC conjugation efficiency was determined by the following calculation:

\[
[IgG] = 3.1 \cdot \frac{A(495)}{[A(280) - 0.31 \cdot A(495)]}
\]  

(7)

Where \(A(495)\) corresponds to excitation/emission wavelength of FITC, and \(A(280)\) corresponds to the excitation/emission wavelength of aromatic ring-containing amino acids.

### 3.2.5 In vitro studies

**Fluorescence microscopy:** To qualitatively determine binding of the MSN-Herceptin conjugate to HER2+ cells, three cell lines were incubated and prepared as previously described (Section 3.1.1). The cell lines (MDA-MB-231(TN), BT474, SKBR3, and HR20) were grown to 95% confluency in 96-well plates. Each cell type was incubated with the MSN-Herceptin-FITC conjugate in DMEM at varying concentrations (50,100,200,400µg/well) and times (30 minutes, 1 hr, 2hr). At the respective time point, all cells were lifted from the well plate by trypsinization. The cell suspension was filtered through a 8µm nylon mesh filter. This would allow for the free MSN to pass, but trap MSN-attached cells on the upstream side of the filter. Similar to MSN release, accumulated cells were detached from the filter by alternating retrograde/prograde flow.

**Flow cytometry:** Preferential cellular uptake of MSN-Herceptin particles were quantified using flow cytometry. The cell lines were prepared in the same manner used for fluorescence microscopy. Data were processed by FloJO.

**Ultrasonography:** To quantitatively show that cellular uptake of MSNs can augment the inherent contrast in B-Mode ultrasound images, a 2D model was applied. After incubating cells in MSN to allow for uptake as previously described for fluorescence microscopy, the recovered cell
suspension was spread across a 10% agar gel. A 5 cm thickness ensured that the focal region of the ultrasound beam would not detect reverberations from the plate bottom. Images were acquired as previously described (Section 3.2.2, “B-mode imaging”)
4. Results

4.1 Mathematical Justification for MSNs as a contrast agent

4.1.1 Contrast by backscatter

By using the Morse-Ingaard model for general ultrasound scatterers, $s$, through a medium, $m$, several comparisons between solid and gas particles can be made:

$$\sigma = N \frac{4\pi}{9} k^4 r^6 \left[ \left( \frac{k_s - k_m}{k_m} \right)^2 + \frac{1}{3} \left( \frac{3(\rho_s - \rho_m)}{2\rho_s + \rho_m} \right)^2 \right]$$

(8)

$\sigma =$ scattering cross section
$N =$ concentration of scatterers per unit volume
$k =$ wave number (equal to $2\pi/\lambda$ of the incident soundwave)
$r =$ particle diameter
$\kappa =$ adiabatic compressibility (inverse of the bulk modulus, $B$)
$\rho =$ density

This model had previously been used to show that gasses exhibit a backscattering cross section around $10^{14}$, whereas solid particles are closer to $2^{[27]}$. The significant disparity in backscatter efficiency of the particle is caused by the exponential relationship to scatterer cross section ($\sigma$) and its compressibility and density terms (bracketed values). Generally it is assumed that $k_m \gg k_g$ and $\rho_m \ll \rho_g$. The Morse model also denotes that backscattering cross section is linearly related to the concentration of scatterers per unit volume. Figure 11 provides an illustrative comparison of acoustic properties of materials relevant to ultrasound imaging. Using soft tissue as the exemplary medium, it is clear that the density and acoustic wave velocity of gasses are much less than soft tissue.
Figure 8. Comparison of Acoustic Properties
If the adiabatic compressibility of a scatterer is not taken into account (such as in equation 2), the disparity in contrast efficiency between gas and solid nanoparticles is now within one order of magnitude. From the figure above, it is clear that silica is denser than soft tissue and has a larger acoustic velocity. Since the differences in density and compressibility are quadratically related to contrast (equation 2), only the absolute difference is critical. For this reason solid particles create contrast despite an inverse relationship to soft tissue, compared to gases. To reiterate, the difference in acoustic impedance between medium and scatterer defines the degree of contrast intensity.

4.2 Characterization of mesoporous silica nanoparticles

TEM imaging (Figure 11) confirmed presence of the hexagonal array typical of MCM-41 type MSNs. Successful TEM imaging proved more difficult than previously anticipated, due to the flocculating nature of the particles. Figure 11.A reveals the irregular form that has been seen as typical for commercially available MCM-41. Due to the nature of TEM imaging, particles were placed on the imaging stage while still in solution. This allowed for cluster formation, as seen in figure 11.A. Prior studies have shown that the degree of aggregation is directly dependent upon concentration [28]. For MCM-41 type MSNS with 300 nm diameters, the critical concentration for an aggregation event to occur was near 500ug/ml.
The irregular macrostructure of the MSNs were further illustrated by SEM (Figure 13). The large macroscale pockets in conjunction with hexagonal nanoscale pores (2.1-2.7nm in diameter) contributes to a surface area ratio of 1000m$^2$/g.

During surface modification steps prior to Herceptin conjugation, FTIR was used to monitor the surface groups (Figure 13). Native MSN, hydroxylated MSN (MSN-OH), and amine-functionalized MSN (MSN-NH2) all exhibited the characteristic peaks of aminosilanes, including H-O-H bending at 1639 cm$^{-1}$ from residual water. The hydroxylated MSN displayed an increase in the broad O-H stretch at 3200-3400 cm$^{-1}$, as expected. Surprisingly, MSN-OH displayed more Si-O-Si bonding than MSN-NH2. This could be due to shielding effect caused by the additional ethoxysilane group seen in MSN-NH2. As expected, MSN-NH2 exhibited peaks at 4000 and 1570 cm$^{-1}$. Any changes in C-H bonding (from APTES) near 3000 cm$^{-1}$ would be masked by the considerably larger residual peaks from the instrument cartridge.
4.3 Nanoparticle concentration to contrast intensity correlation

In order to determine the image contrast due to MSN, acrylamide plugs were polymerized with varying levels of MSN concentration and imaged using conventional clinical ultrasound (Figure 14, 15). Image contrast never approached a mean pixel intensity (MPI) of 0 since anomalous reflections were detected from imperfections in the acrylamide plug. The correlation of contrast to MSN concentration is linearly related, as expected from the Morse-Ingaard model (Equation (8)): The increase of scatterers, \( N \), is directly proportional to the scattering cross section, \( \sigma \). The Morse-Ingaard model also predicts scattering cross section to have a fourth order dependence on the transducer probe frequency (ie. the wavenumber, \( k \), in units of Hertz). As frequency increases, the backscatter cross section increases exponentially. For this set of experiments, imaging was limited to a probe with a central frequency of 7.5 MHz with a maximum limit of 10MHz.
Exploring higher frequencies was limited by availability of probe types. In a clinical setting, ultrasound probes may operate at a range between 2-18 MHz.

In the traditional sense, axial resolution (or longitudinal resolution) is defined as the detectable distance between two objects on a line parallel to the direction of wave propagation. Axial resolution can be calculated as half of the spatial pulse length, SPL. SPL is defined as the wavelength, $\lambda$, multiplied by the number of cycles, $\delta$, emitted per pulse. Wavelength can be further decomposed as a product of acoustic velocity, $c$, and the frequency of the incident sound wave, $f$. Using the experimental setup for determining the correlation of MSN concentration to MPI ($f = 7.5$ MHz, $c = 1540$ m/s, $\delta = 1$), the theoretical axial resolution can be calculated:

$$R_{axial} = \frac{SPL}{2} = \frac{\lambda \cdot \delta}{2} = \frac{c \cdot \delta}{2f}$$ (9)

$$75\mu m = \frac{1540 m/s^{-1} \cdot 1}{7.5 \cdot 10^6 s^{-1} \cdot 2}$$
Clearly the contrast apparent in Figure 14 is significantly smaller than the theoretical axial resolution, with a diameter closer to $1/10^{th}$ of the projected resolution. Considering the drastic disparity between these two values, the apparent contrast is not caused by creating an image of the individual particle boundary, but rather increasing the inhomogeneity of regions in the microscale. In essence, MSN particles increase the “speckle” noise within a medium. Although contrast does not reflect the underlying structure of these sub-resolution scatterers, it does reflect their echogenicity\cite{29}. As described previously (Section 4.1.1), the inherent density and acoustic velocity of silica material in soft tissue yields a substantial degree of reflectivity.

Convention may suggest that increasing speckle noise can decrease the visibility of a boundary region, as the case with certain hypoechoic lesions. The contrast caused by a sub-resolution scatterer is calculated by statistical probability. Each scatterer creates a random phasor along a complex plane (Figure 16). A phasor is a representation of a sinusoidal function. In this case, the function is the response to an incident sound wave. The contrast of a region can be defined as the vector sum, $r$, of each individual scatterer (Figure 16, left). In uniform medium, when each scatterer population, $r$, is compiled with scatterer
populations in the same region, the amplitude of contrast within the region equates to a 2D Gaussian distribution (Figure 16, right). Reverting back to optics theory, this can also be defined as a Rayleigh probability distribution function (PSD). Since all scatterer population cancel each other out, a zero-mean phasor results, and any contrast is observed as noise. However, by targeting the silica particles to specific regions of congregation – through flocculation in phantoms or Herceptin receptor targeting in vitro – the periodic arrangement creates a coherence component that is added to the speckle noise statistics. By adding a coherence component to a sub-resolution scatterer, a strong phasor is added to the system. This results in a shift of the PSD either to the right or left along the real axis. Since scatterer arrangement at the lesion boundary (or flocculation cluster) would not be random, contrast can be detected despite having a sub-resolution diameter. By having an “ordered arrangement of speckle”, contrast of tumors at the boundary layer can be accentuated.

4.4 Cellular interaction of nanoparticles

4.4.1 Fluorescence microscopy

A preliminary view of HER2-positive and HER2-negative cell lines reveals a modest yet detectable discrimination between the two cell types (Figure 14). While the MSN-Herceptin nanoparticles are present in the filtered HER- culture (14.A), most of the particles are either unbound or attached to the outer wall of the cell membrane. Conversely, most HER2+ cells (14.B) show attachment of the nanoparticles to the cell. The data does not confirm the precise location (internal or external) of particle attachment to the cell. Both cell populations were
sieved after particle incubation using a 10µm nylon mesh filter, theoretically eliminating all unbound particles whose diameter is less than 10µm.

4.4.2 Flow cytometry

A FACSCalibur flow cytometer was used to determine the binding of the MSN-Herceptin-FITC nanoparticle to HER+ cells, as well as determine the cell viability after incubation. Since compensation was not achievable, the two experiments were not conducted simultaneously.

The binding affinity of the nanoparticles were characterized by the relative absorbance

![Image of flow cytometry data]

Figure 15 Comparison of cells treated with MSN-Herceptin-FITC (A) HER- cells: 231, (B) HER+ cells: skbr3

![Image of sample gating for flow cytometry]

Figure 16. Sample gating of cells for flow cytometry. Shown: 231 treated with MSN-Herceptin
near FITC’s maximum emission wavelength of 520 nm, using a 530/30 bandpass filter. The FITC-conjugated molecules were excited using a 488nm laser, and polygon-gated to capture the event population with a larger forward and side scatter (Figure 15). This gating only captures approximately 40% of the event population as a precautionary measure to exclude debris, unbound residual particles, or dead cells (for the binding assay experiment only).

Following gating, FITC fluorescence was measured for each cell type (Figure 15, right). The control skbr3 and 231 yielded nearly identical profiles, as expected (231 group not shown). The MSN-treated 231 cell population (HER2 positive) expressed a detectable degree of fluorescence from the FITC particles; any fluorescence should indicate any HER2 receptor expression on the surface. The control skbr3 and 231 yielded nearly identical profiles, as expected (231 group not shown). The MSN-treated 231 cell population (HER2 positive) expressed a detectable degree of fluorescence from the FITC particles; any fluorescence should indicate any HER2 receptor expression on the surface. The MDA-MB-231(TN) cell line has been characterized as either a basal-like and triple negative breast cancer (TNBC) line in literature. Groups such as B. Lehmann et al. define these TNBC lines as “lacking estrogen receptor (ER) and progesterone receptor (PR) expression as well as human epidermal growth factor receptor 2 (HER2) amplification”. A normal, non-amplified level of HER2 expression may account for the apparent binding of MSN-Herceptin-FITC particles to this cell type. Alternatively, indiscriminate binding may be caused by the silica particle itself, as opposed to the Herceptin antibody. Trewyn et al. have shown than nude MSNs are naturally incorporated by certain cell types. The accelerated metabolism of cancer cells increase their cellular uptake, consequentially reducing their partiality for nutrients. In a qualitative sense, the rate of cell division far exceeds both skbr3 and skbr3pool.

![Figure 17. Dead cells ratio comparison using ethidium homodimer 1](image-url)
Skbr3, which indeed overexpresses HER2, shows a strong binding affinity with the nanoparticles. From an arbitrary cutoff near $10^2$ fluorescence, 96.2% of the gated events from the skbr3 cell line exceed that cutoff. Alternatively, the 231 cell line exhibits only 15.1% of the gated events pass the cutoff.

Since silica yields a wide absorption spectra, UV-VIS spectroscopy was skewed by the concentration of MSNs. For this reason an MTT assay to determine cell proliferation was not possible (Appendix D). Alternatively, ethidium homodimer-1 (EthD-1) was used to determine the ratio of dead cells to total cell count. By using a 488nm laser and a 650nm long pass filter, the tail of the EthD-1 emission profile could be observed without nearly any overlap from FITC’s emission spectra (Appendix E). EthD-1 interference for the binding affinity experiment was not an issue since those cells were not treated with EthD-1.

A comparison of the fluorescence of skbr3 cells with and without MSN treatment suggest that the MSN treatment reduces overall cell proliferation. A shift in fluorescence caused by EthD-1 uptake indicates that the Herceptin targeting arm also maintains a certain degree of its therapeutic capability. However, further testing is required to confirm this claim.

### 4.4.3 in vitro ultrasound imaging

After incubation in MSN-Herceptin, cells were lifted of the well plate and aliquot onto agar inside of a 5 ml syringe. After allowing 2 hours for the cells to settle to the agar floor, most of the media was pipetted off. A second layer of agar was poured over to the remaining cell suspension (Figure 17). A cross section of the sandwiched cell culture was imaged on a clinical ultrasound machine at 10MHz. To quantify the peak pixel intensity and thickness of contrast, the pixel intensity profile along the y dimension was graphed (yellow line, Figure 17). Five profiles with variable x coordinated were averaged to produce the mean pixel intensity profile (Figure 17, bottom).
The presence of MSN-Herceptin results in a spike in contrast by approximately 640%, with a boundary thickness close to 1mm. The total cell count in the suspension is estimated to be around 200,000 cells spread across an area of 122mm².

A comparison of the two cell lines – 231 and skbr3 – suggests that there is minimal and insignificant discrepancy in contrast. This observation is partially in conflict with the binding affinity assay from flow cytometry, which suggests that there is a significant difference between the binding affinities of MSN-Herceptin to cells that do and do not overexpress the HER2 receptor. This observation is also supported indirectly by UV-VIS spectrometry (Appendix D). The binding affinity assay does indicate, however, that there is a certain degree of binding to all cell types. Figure 19 suggests that this indiscriminate binding is enough to create contrast nearly equivalent to the HER2+ cell line. Possible explanations for this occurrence is provided in the previous section.
Figure 19. Contrast profile cross sections of HER2+ and HER2- cells treated with MSN-Herceptin
5. **Discussion**

5.1 **Contrast Efficacy**

The potential for silica nanoparticles to act as an effective contrast agent relies on the delivery and retention method of the particles. It has been well proven that gas phase contrast agents are ideal for contrast enhancement in dynamic systems (such as cardiovascular flow). Although silica nanoparticles do provide some degree of contrast in dynamic systems, it is most effective in static systems. By operating in a static system, the silica nanoparticles can form what may be termed as a flocculation layer. The formation of this deposition layer is requisite for both its drug delivery functionality as well as its contrast agent functionality. Due to the relatively small diameter of the MSN particle with respect to the ultrasonic wavelength, Rayleigh-type backscatter is diminished. By having substantially high concentrations in at tumor sites, ultrasound contrast can be augmented.

Point scatterer concentration may fluctuate greatly in dynamic systems, where concentration is constantly modified by directional flow. Due to a heavy influence from dispersive flow movement, gasses-filled microbubbles typically only display a half-life in the region of interest up to a few minutes. This time is further reduced by the rupturing potential of ultrasound waves. One allure of antibody-conjugated silica contrast agent is that it is extremely stable in comparison to liposome-enclosed air bubbles, thus eliminating issues of half-life. By chemically attaching the silica particles to the region of interest the concentration is further stabilized.

Due to the high porosity of mesoporous silica, air bubbles may be trapped within the core of the nanoparticle. Part of the contrast effect may be a product of reflectivity enhancement that occurs by backscatter from these air bubbles trapped in the pores. With a pore volume ranging from 0.98 cm$^3$/g (MCM-41) up to 2.31 cm$^3$/g (MSU-F), it is possible for air pockets to comprise more than 33% of the bulk volume (MCM-41). However, the air retention with the particles was not observed in this study.

Flow cytometry and in vitro ultrasound indicate that the preferential binding of MSN-Herceptin particles to the different cell types is significant, but not optimal for cell-specific ultrasound contrast enhancement. One proposed theory is the indiscriminate uptake of silica particles by numerous cell types. By increasing the count of Herceptin targeting arms on the surface, more specific binding may be achieved. Since each Herceptin molecule is conjugated to
the amine group of the APTES precursor at a 1:1 ratio, the density of Herceptin targeting arms on the silica surface is limited by the geometric area occupied by the APTES base. Assuming that the triethoxysilane base is $3.96 \times 10^4 \text{ pm}^2$ and the surface area to mass ratio of MCM-41 silica is $1000 \text{ m}^2/\text{g}$ (provided by Sigma), the limit of Herceptin bound to the silica surface is 0.041 moles per gram of silica.

5.2 Potential Applications

Currently, ultrasound is generally used for two purposes in the context of breast cancer detection and treatment: secondary diagnosis and presurgical occult lesion localization (Section 1.1, ‘Review of breast cancer ultrasound diagnostics’). In both cases, the efficacy of the procedure is heavily reliant on its ability to discern tumor boundary regions.

In a clinical setting, ultrasound has typically been used as a complementary diagnostic device with mammography. The greatest disadvantages of mammography is that it is a radiation-emitting modality and that it is less effective than ultrasound for density determination. However, even ultrasound is unable to distinguish between benign and malignant tumors of similar densities. Ultrasound specificity is further diminished if the surrounding tissue is similar in density, which is common for women below 50 years old. By injecting MSN+Herceptin particles in tissues regions suspected of cancerous lesions, contrast enhancement will only occur in cancerous tumor regions. By being able to visualize selective contrast variations in real time, sonographic images can more effectively distinguish malignant tumors from benign cysts.

Ultrasound is also commonly used for needle guidance, both for biopsy sampling and occult lesion localization. If the boundary region of a suspected tumor is difficult to discern, the result may be poor sampling localization or poor marker placement. By improving visualization of the boundary regions, biopsy needle placement can be more accurate, or rendered unnecessary altogether. Since wire length for occult lesion localization is determined by the length of the lesion, improved visualization of the boundary regions can ensure that the wire completely passes through the entirety of the lesion.
### Table 2. Advantages and disadvantages of silica nanoparticles as a contrast agent in comparison to gas phase contrast agents

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Better suited for extravasation out of leaky tumor capillaries</td>
<td>• Lower scattering cross section than microbubbles</td>
</tr>
<tr>
<td>• More stable, allowing longer half-life in body to take multiple images</td>
<td>• Size approaches axial resolution of conventional ultrasound transducer frequencies</td>
</tr>
<tr>
<td>• Highly modifiable surface</td>
<td></td>
</tr>
<tr>
<td>• Potential for reflectivity enhancement</td>
<td></td>
</tr>
<tr>
<td>• Large pores allow for delivery of low solubility drugs</td>
<td></td>
</tr>
<tr>
<td>• US: portable and cheaper than Mammography</td>
<td></td>
</tr>
<tr>
<td>• FDA approved</td>
<td></td>
</tr>
<tr>
<td>• Permanent retention within cell possible (depends on cell type)</td>
<td></td>
</tr>
</tbody>
</table>
6. Conclusion

6.1. Conclusion

Observation of the acoustic properties of 1µm unfunctionalized mesoporous silica nanoparticles (MSNs) revealed that the backscatter coefficient of these particles are sufficient to be considered as an ultrasound enhanced contrast agent (UECA). By conjugating MSN to the HER2-targetting antibody Herceptin (trastuzumab), local concentrations near tumor sites overexpressing HER2 receptors increased and further improved the backscatter cross-section of the MSNs. In vitro studies suggest the binding affinity of the MSN-Herceptin particles is preferential to cancer cells that overexpress the HER2 surface receptor. Due to the large size of the particle conjugate, the particles are not incorporated into the cell by endocytosis. Targeted MSN are ideal for ultrasound contrast enhancement, ultimately improving the reliability of cheaper, more user friendly, and non-radiating imaging modality.

6.2 Future Direction

6.2.1 In vivo studies

The next objective of the project is to prove contrast enhancement in vivo. Nude athymic BALB/c female mice will be used for a tumor xenograft model. The breast cancer cell lines used for in vitro studies will be cultured and subcutaneously injected into the right flank of 4-6 week old nude mice. After tumor formation up to 65mm³, the mice will be given intraperitoneal injections of MSN+Herceptin+FITC. Following injection, B-mode images will be taken at the tumor site using a 40MHz transducer probe. Prior to study, LD50 of MSN+Herceptin and the final endpoint of the study must be determined. Injection frequency and ultrasound imaging is dependent upon cellular uptake and filtration time.

6.2.2 MSN as a theranostic agent

A great deal of research has been dedicated towards the application of MSN as a drug delivery vehicle [30]. However, the innate properties of MSN also allow them to be applied as an agent recently termed as a theranostic – the combination of therapeutics and diagnostics into a single agent. Already MSNs have shown their potential as a therapeutic agent (Section 1.4.2, ‘Biomedical Applications of MSNs’). As evidence for MSNs as a UECA builds, these particles will increasingly become more appealing as a theranostic agent as well.
The current conjugation motif of MSN+Herceptin is already in essence a theranostic agent: the original purpose of Herceptin is not to act as a targeting agent, but rather to act as a treatment for a HER2+ breast cancer types. Further study of cell proliferation under MSN+Herceptin inoculation must be taken to determine how MSN conjugation affects the therapeutic efficacy of Herceptin. It is worth noting that the conjugation motif of this study did not include any form of drug loading into the cells; this may provide a second ‘double punch’ for its therapeutic characteristics.

6.2.3 Incorporating perfluoropentane into the MSN pores

A high degree of porosity allows the MSN particle cavity to be loaded with material and used as a carrying agent. Similar to drug loading, MSNs may also be loaded with a gas. The introduction of a gas phase would theoretically enhance the backscatter coefficient in a way similar to microbubbles (solid-to-gas or liquid-to-gas interfaces yield large backscatter). The rigidity of the MSN pores may restrict the oscillatory motion typically seen in microbubble contrast agents. Alternatively, this restriction of motion will also provide for greater stability and half-life. As mentioned previously, extended half-life is beneficial for long term tumor tracking or extended imaging time.

Like drug loading, gas retention within the particles may be solved by capping. Several groups have applied hard caps (iron oxide Fe$_3$O$_4$, cadmium sulfide CdS, gold Au) as well as soft caps (G-Insulin)\textsuperscript{[31]}. Most of these capping agents can be removed by environmental stimuli. Fortunately cap release may not be necessary, thus sidestepping a major issue with this field of research. However, cap release under cancer cell stimuli may have an advantage as well. Heavy gasses such as perfluoropentane are used in most clinically approved contrast agents due to their low partition coefficient in blood. By incorporating a gas phase into MSNs, backscatter may be drastically increased.

6.2.4 Dye loading for presurgical occult lesion localization

Currently, wire placement is the standard for tumor marking prior to surgery. This method is inefficient, since only one dimension of the tumor is marked for surgical removal. Consequentially, an excessive amount of tissue around the lesion must be removed as a precautionary measure. By functionalizing the surface or loading the MSN pores with a slow-release dye, targeted cells can be easily stained. Upon scission of the breast during surgery,
stained cancer cells can easily be visualized and removed. Dyes such as cyanine can be easily excited under a blacklight lamp (low energy UVA).
APPENDIX

A. MATLAB code: Single-Pulse Processing

```matlab
% 1D Signal Processing Protocol
% By: Andrew Milgroom
% Translational Biomaterials Research Labs
% University of Colorado- Anschutz Medical Campus
% Date: 09/17/2012

% This protocol describes the attenuation of an ultrasound signal by
% deconvoluting the image with a signal through pure water through the same
% distance.

% Set variables, figure dimensions, and get screen size
signal = [];
signalAUC = 0;
signalDC = [];
water = [];
fx = 1000;
fy = 600;
Size = get(0,'screensize');

% Create the figure and center it on the screen
handles.hfig2 = figure('NumberTitle','off',
... 'Position',[(floor((Size(3)-fx)/2) 
... floor((Size(4)-fy)/2) 
... fx fy),... 'Name','Single Pulse Processing GUI',...
... 'Color',[1 1 1],...
... 'PaperPositionMode','auto',...
... 'DoubleBuffer','On',...
... 'MenuBar','none');

% Create file menu, graph window, data box and buttons
menu = uimenu(handles.hfig2,'Label','File');
   uimenu(menu,'Label','Load Image...','Callback','[signal water] =
... loadsignal([signal,water]);
   uimenu(menu,'Label','Close','Callback','close');
aucbox = uicontrol(handles.hfig2,'Style','edit','Units','Normalized','Position',...
... [.05 .35 .2 .07],'Tag','(aucbox);'
auctitle = uicontrol(handles.hfig2,'Style','text','Units','Normalized','Position',...
... [.05 .43 .2 .03],'String','Cumulative Voltage Difference');
grptitle = uicontrol(handles.hfig2,'Style','text','Units','Normalized','Position',...
... [.55 .53 .15 .03],'String','Signals');
sigtitle = uicontrol(handles.hfig2,'Style','text','Units','Normalized','Position',...
... [.1 0.96 .15 .03],'String','Signal');
watertitle = uicontrol(handles.hfig2,'Style','text','Units','Normalized','Position',...
... [.4 0.96 .15 .03],'String','Water');
dctitle = uicontrol(handles.hfig2,'Style','text','Units','Normalized','Position',...
... [.7 0.96 .15 .03],'String','Deconvoluted signal');
denoisebtn = uicontrol(handles.hfig2,'Style','PushButton','Units','Normalized','Position',...
... [.07 .26 .15 .05],'String','Denoise and Smooth','Callback', '[signal water] =
... denoise(signal water);');
analysisbtn = uicontrol(handles.hfig2,'Style','PushButton','Units','Normalized','Position',...
... [.07 .19 .15 .05],'String','Run Analysis','Callback', 'signalAUC signalDC =
... analyze(signal water signalAUC signalDC);');
```

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savebtn = uicontrol(handles.hfig2, 'Style', 'PushButton', 'Units', 'Normalized', 'Position', [.07 .12 .15 .05], 'String', 'Save to file', 'Callback', 'Data = save(signal signalAUC signalDC);');

handles.mainaxes = axes('Parent', handles.hfig2);
axes(handles.mainaxes);
set(gca, 'Title', text('String', ''));
set(handles.mainaxes, 'XTickLabel', []);
set(handles.mainaxes, 'FontSize', 8, 'Color', [1 1 1], 'Box', 'On', 'YColor', [0 0 0], 'XColor', [0 0 0], 'XTickLabel', [], 'Units', 'Normalized', 'Position', [.3 .12 .6 .4]);

handles.signalaxes = axes('Parent', handles.hfig2);
axes(handles.signalaxes);
set(gca, 'Title', text('String', ''));
set(handles.signalaxes, 'XTickLabel', []);
set(handles.signalaxes, 'FontSize', 8, 'Color', [1 1 1], 'Box', 'On', 'YColor', [0 0 0], 'XColor', [0 0 0], 'XTickLabel', [], 'Units', 'Normalized', 'Position', [.05 .6 .25 .35]);

handles.wateraxes = axes('Parent', handles.hfig2);
axes(handles.wateraxes);
set(gca, 'Title', text('String', ''));
set(handles.wateraxes, 'XTickLabel', []);
set(handles.wateraxes, 'FontSize', 8, 'Color', [1 1 1], 'Box', 'On', 'YColor', [0 0 0], 'XColor', [0 0 0], 'XTickLabel', [], 'Units', 'Normalized', 'Position', [.35 .6 .25 .35]);

handles.dcaxes = axes('Parent', handles.hfig2);
axes(handles.dcaxes);
set(gca, 'Title', text('String', ''));
set(handles.dcaxes, 'XTickLabel', []);
set(handles.dcaxes, 'FontSize', 8, 'Color', [1 1 1], 'Box', 'On', 'YColor', [0 0 0], 'XColor', [0 0 0], 'XTickLabel', [], 'Units', 'Normalized', 'Position', [.65 .6 .25 .35]);

function [signal water] = loadsignal(signal,water)
% This function loads the signal from the user, and grabs the water file % from a previously described path

function [signal water] = denoise(signal water)
% This function denoises AND smooths the signals

function signalAUC signalDC = analyze(signal water signalAUC signalDC)
% This function analyzes the smoothed and denoised signal by deconvoluting % it and looking at the area difference between water and the signal. The % the display for 'DC' will appear
function Data = save(signal signalAUC signalDC)
% This function saves the original signal, the cumulative difference (AUC)
% and the deconvoluted signal. The data is added to a previously described
% workspace
B. MATLAB code: B-mode image processing

```matlab
% B-Mode Image Processing Protocol
% By: Andrew Milgroom
% Translational Biomaterials Research Labs
% University of Colorado- Anschutz Medical Campus
% Date: 08/01/2012

% This protocol imports a grayscale image of a B-Mode ultrasound scan from
% selecte filepath. The image automatically detects the boundary of the
% region of interest (ROI). The region of interest is parsed into 50 pixel
% x 50 pixel squares. For each square, mean pixel intensity distribution
% (w/st. dev.) and 2D Fourier transform is acquired. All squares are then
% averaged and saved to a predefined workspace, where it can be compared
% with other data

% Set variables, figure dimensions, and get screen size
Bscan = []; tempdata = []; fx = 800; fy = 600; Size = get(0, 'screensize');

% Create the figure and center it on the screen
handles.hfig2 = figure('NumberTitle', 'off', ...
    'Position', [floor((Size(3) - fx)/2) ...
    floor((Size(4) - fy)/2) ...
    fx fy],...
    'Name', 'B-Mode Image Processing GUI', 'Color', [1 1 1],...
    'PaperPositionMode', 'auto', 'InvertHardcopy', 'off',...
    'MenuBar', 'none');

% Create file menu, graph window, data box and buttons
menu = uimenu(handles.hfig2, 'Label', 'File'); uimenu(menu, 'Label', 'Load Image...', 'Callback', 'Bscan = loadimage(Bscan)'); uimenu(menu, 'Label', 'Close', 'Callback', 'close');

mgsbox = uicontrol(handles.hfig2, 'Style', 'edit', 'Units', 'Normalized', 'Position', ...
    [.05 .85 .2 .07], 'Tag', 'mgsbox');

stdbox = uicontrol(handles.hfig2, 'Style', 'edit', 'Units', 'Normalized', 'Position', ...
    [.05 .70 .2 .07], 'Tag', 'stdbox');

mgstitle = uicontrol(handles.hfig2, 'Style', 'text', 'Units', 'Normalized', 'Position', ...
    [.08 0.93 .15 .03], 'String', 'Mean Gray Scale');

stdtitle = uicontrol(handles.hfig2, 'Style', 'text', 'Units', 'Normalized', 'Position', ...
    [.08 0.78 .15 .03], 'String', 'Standard Deviation');

bscantitle = uicontrol(handles.hfig2, 'Style', 'text', 'Units', 'Normalized', 'Position', ...
    [.55 0.93 .15 .03], 'String', 'B-Mode Image');

analysisbtn = uicontrol(handles.hfig2, 'Style', 'PushButton', 'Units', 'Normalized', 'Position', ...
    [.07 .19 .15 .05], 'String', 'Run Analysis', 'Callback', 'tempdata = analyze(Mtrace,Atrace,Bscan,tempdata);');

savebtn = uicontrol(handles.hfig2, 'Style', 'PushButton', 'Units', 'Normalized', 'Position', ...
    [.07 .12 .15 .05], 'String', 'Save to file', 'Callback', 'Data = save(Data,tempdata);');

handles.mainaxes = axes('Parent', handles.hfig2); axes(handles.mainaxes);
set(gca, 'Title', text('String', ''));
```

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function Bscan = loadimage(Bscan)
% Loads image from JPG or TIFF file, saves image as matrix, 'image', and
% displays it on the screen
Bscan = uigetfile
imshow(Bscan)

function tempdata = analyze(Mtrace,Atrace,Bscan,tempdata)
% Finds mean grayscale pixel intensity, its distribution, as well as the
% fourier transform of the image (currently unused). Avoids bias by
% sampling the ROI in 50 pixel x 50 pixel boxes then averages the values.
% Total box count varies.

function Data = save(Data, tempdata)
% Adds new image data to the previous workspace
C. Herceptin Protein sequence

Human trastuzumab peptide sequence

>Anti-HER2 Light chain 1
DIQMTQSPSSLASVGDRVITITCRASQDVNTAVAWYQQKPGKAPKLILLYSASFLYSVGSVPSSFGRSGSDFTLTILLQPEDFATYQQHYYIYHTPMPTFGQGTKVEIKRTVAAAPSVIFPFPSEQLKGSATASVVCNNFYQPREAKVQMKVDNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEHKVKYACEVTHQGLSSPVTLSFPRNGEC

>Anti-HER2 Heavy chain 1
EVQLVESGGGLVQPGGLSLSLCTAAAGFVSKDYIGEMYSRWFQGTVTVSASTKGPSVFPLAPSSKSTSGTALALGVKLTVQAVPGKGLEWVQIYVQEWQFETNGYSRATSVKGRFTISADSDKIIEQNLKDVEKDDIKGRFVTCKPRGQPPQVYTLPPSRDELTQKVSLTCLVGYFSPDIAVEWESNGQPENNYKTVPSVLDGLSFLYSKLTVDKSRWQQGNVFLCSCVMHEALHNHYTQSKLSLSPGK

>Anti-HER2 Light chain 2
DIQMTQSPSSLASVGDRVITITCRASQDVNTAVAWYQQKPGKAPKLILLYSASFLYSVGSVPSSFGRSGSDFTLTILLQPEDFATYQQHYYIYHTPMPTFGQGTKVEIKRTVAAAPSVIFPFPSEQLKGSATASVVCNNFYQPREAKVQMKVDNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEHKVKYACEVTHQGLSSPVTLSFPRNGEC

>Anti-HER2 Heavy chain 2
EVQLVESGGGLVQPGGLSLSLCTAAAGFVSKDYIGEMYSRWFQGTVTVSASTKGPSVFPLAPSSKSTSGTALALGVKLTVQAVPGKGLEWVQIYVQEWQFETNGYSRATSVKGRFTISADSDKIIEQNLKDVEKDDIKGRFVTCKPRGQPPQVYTLPPSRDELTQKVSLTCLVGYFSPDIAVEWESNGQPENNYKTVPSVLDGLSFLYSKLTVDKSRWQQGNVFLCSCVMHEALHNHYTQSKLSLSPGK

Total AA count: 1,330
Molecular Weight 145531.5 g/mol

Lysine: 90/1,330 (6.77%)
Aspartic Acid: 58/1,330 (4.36%)
Glumatic Acid: 60/1,330 (4.51%)
Amines (plus termini): 7.07% 10,285.7 g/mol
Carboxylates (plus termini): 9.17% 13,349.5 g/mol
D. UV-VIS spectroscopy

Cells were incubated overnight at 37°C in 0.125 mg/ml MSN-Herceptin. Afterwards the cells were washed, trypsinized, and sieved with a 10µm nylon mesh. The samples were treated with 10µl of 12mM MTT solution for 30 minutes at room temperature. Absorbance at 540nm was measured (top figure). The graph below suggests that not only does the MSN-Herceptin induce cell proliferation with respect to HER2 expression, but may increase proliferation beyond the untreated samples. A more plausible explanation would be that the MSN has a stronger binding affinity to HER2+ cells. As MSN concentration increases, more light is absorbed in the well. Theoretically, retention of 0.025 mg in a 100µl volume could increase absorbance by ~0.07 (bottom figure).
E. FITC and EthD-1 excitation and emission spectra

![Excitation and emission spectra of 7-aminoactinomycin-D](image)

Excitation and emission spectra of 7-aminoactinomycin-D
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