Targeted Gold Nanoparticles for the Computed Tomography Detection of Breast Cancer

a Master Thesis
by
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Abstract

Targeted Gold Nanoparticles for the Computed Tomography Detection of Breast Cancer

Introduction: As a response to the need for earlier detection of malignant tissues and metastases, functional imaging has been gaining interest over structural imaging. Gold has proven to be an attractive alternative to conventional contrast agents, with a higher atomic number and a higher absorption coefficient compared to iodine. Due to these properties, gold nanoparticles (AuNPs) could offer a 3-fold increase in contrast per unit weight compared to iodine-based contrast agents, as well as a better safety profile compared to iodine or Gadolinium-based agents in terms of renal and cutaneous toxicity.

Methods: Breast cancer cells MDA-MB-231, macrophages RAW 264.7 and fibroblasts MC3T3.E1 were scanned using a pre-clinical CT to analyze gold nanoparticle uptake. The cells were analyzed after incubation with a AuNPs solution for four hours and silver staining using light microscopy.

Results: Breast cancer cells showed the most intense signal for 2-DG-AuNPs (916 HU), and 2-NH2-DG-AuNPs (435 HU), with almost no signal registered for the bare AuNPs, at 22 HU as opposed to 19 HU for the control sample.

Conclusion: Functionalized 2-DG-AuNPs were a valid targeting contrast agent for cell lines MDA-MB-231 and RAW 264.7, exhibiting high radiointensity upon CT imaging and low toxicity.

Nederlandse samenvatting

Gerichte Gouden Nanodeeltjes voor de Computertomografie Detectie van Borstkanker

Inleiding: Als reactie op de behoefte naar eerdere detectie van kwaadaardige weefsels en metastasen, is functionele beeldvorming belangrijker dan structurele beeldvorming aan het worden. Goud heeft bewezen een aantrekkelijk alternatief te zijn voor conventionele contrastmiddelen, met een hoger atoomgewicht en een hogere absorptiecoëfficiënt in vergelijking met jodium. Door deze eigenschappen kunnen gouden nanodeeltjes (AuNPs) een 3-voudige toename van het contrast per gewichtseenheid bieden in vergelijking met op basis van jodium gebaseerde contrastmiddelen, evenals een beter veiligheidsprofiel ten opzichte van iodine of Gadolinium-gebaseerde agens in termen van nier en cutane toxiciteit.

Methoden: Borstkancercellen MDA-MB-231, macrofagen RAW 264.7 en fibroblasten MC3T3.E1 werden gescand met behulp van een preclinische CT om de opname van gouden nanodeeltjes te analyseren. De cellen werden geanalyseerd na incubatie met een AuNPs oplossing gedurende vier uur en zilverkleuring met behulp van licht microscopie.

Resultaten: Borstkancercellen vertoonden het meest intense signaal voor 2-DG-AuNP's (916 HU) en 2-NH2-DG-AuNPs (435 HU), met bijna geen signaal geregistreerd voor de blote AuNPs, bij 22 HU in tegenstelling tot 19 HU voor het controle monster.

Conclusie: Gefunctionaliseerde 2-DG-AuNPs waren een geldig targeting contrastmiddel voor celllijnen MDA-MB-231 en RAW 264.7, die hoge radiointensiteit vertoonden bij CT-beeldvorming en lage toxiciteit.
Introduction

Breast cancer is the second most commonly diagnosed type of cancer worldwide and one of the leading causes of death in women in developed countries, second only to lung cancer (1,2). Although the overall 5-year survival rate in patients with breast cancer has improved over the past decades, the disease is still a serious public health threat (3,4). More than 3.5 million women in the United States are living with a history of breast cancer and in 2014 approximately 41,213 deaths caused by this type of cancer were registered (1,2,5). Despite the undeniable concern posed by this disease, recent advancements in screening and treatment techniques, such as surgery, radiotherapy and adjuvant systemic therapy (endocrine therapy, chemotherapy with anthracyclines and taxanes) have brought little improvement to the life expectancy and quality of life of breast cancer patients (5).

The golden standard for breast cancer imaging is mammography (6). Early detection and diagnosis strongly correlate with a better outcome and higher survival rates and there is evidence that mammography screening could reduce breast cancer mortality rates in several countries (6,7). However, in women with dense breasts, the sensitivity of mammography is only 62.9% and the specificity is 89.1%, as opposed to 87% sensitivity and 96.9% specificity in women with fatty breasts. Younger women are more likely to have dense breast tissue and cancer lesions are therefore harder to diagnose with conventional mammography in this patient group (6-8). Moreover, mammography has a sensitivity of only 55-68% for the diagnosis of locally recurrent breast cancer. This is particularly due to the post-operative benign changes that appear after breast conserving surgery (BCS), such as edema, calcifications, asymmetry and skin thickening (8,9). Another disadvantage would be the discomfort caused by the compression of the breasts and the exposure to high doses of ionized radiation (8).

Standard clinical imaging techniques such as mammography, ultrasound and MRI are classified as structural imaging modalities because although they can readily identify anatomical patterns and tumor location and size, they cannot distinguish between benign and malignant lesions and are not able to detect metastases smaller than 0.5 cm (10). Due to these limitations, a new field of imaging has been raising interest in recent years: nanoimaging (11,12). Nanoimaging is one of the five branches of nanomedicine, together with nanomaterials, analytical tools, novel therapeutics and clinical and toxicological issues. Nanomedicine represents the use of molecular tools and molecular knowledge of the human body to diagnose, treat and prevent disease (12). One of these molecular tools are nanoparticles (NPs).

Nanoparticles have unique physiological, optical and magnetic characteristics which bring new possibilities to the field of imaging (13,14). Their size usually ranges between 1-100 nm and they can have different properties depending on shape, size and surface chemistry (10,15,16). They can be used for imaging specific receptors, vascular abnormalities and even innate immune responses (15). Because nanoparticles have a large surface area per unit of mass (over 1000 m²/g), they are more reactive than macro particles. This is because in smaller molecules, most atoms tend to reside on the surface of the particle, increasing reactivity (16).

One of the most researched types of nanomaterials are gold nanoparticles (AuNPs) (16-18). Unlike bulk gold, which is of course gold in color, nanoscale gold can exhibit vivid colors ranging from red, blue and green to brown and almost black (19,20). Such colors can arise from interaction between the electric field vector of incident light and the conduction band electrons of the metallic nanoparticles (19,21). The word “nano” is derived from Greek (nanos) and can be used to describe materials with dimensions between 1-100 nm. Unlike smaller molecules, gold nanoparticles enter cells by faster mechanisms and accumulate at
tumor or inflammatory sites (20). Moreover, they can be used as artificial antibodies due to their facile surface chemistry, or to increase imaging contrast in diagnostic CT (computed tomography) (20,22,23).

Computed Tomography is one of the most common imaging modalities in hospitals as a diagnostic tool, having the advantages of providing superior tissue penetration and spatial resolution (6,24,25). CT works by visualizing differences between tissue densities, which results in clear anatomical images. However, in order to see the differences between diseased and normal tissues, specific contrast agents need to be used. Radiopaque X-ray contrast agents are usually injected intravenously and exhibit non-specific penetration and binding, as well as rapid renal excretion (26). These limitations have determined a surge in the research of targeting agents and cellular imaging, using metal-based agents instead of the traditional iodinated ones, such as Omnipaque or Visipaque (24,25,27).

Gold has proven to be an attractive alternative to conventional contrast agents, having a higher atomic number and a higher absorption coefficient compared to iodine. Due to these properties, gold nanoparticles offer a 3-fold increase in contrast per unit weight compared to iodine-based contrast agents, as well as a better safety profile compared to iodine or Gadolinium-based agents in terms of renal and cutaneous toxicity (25,26,28). Moreover, imaging gold nanoparticles at 80-100 Kiloelectron Volts provides lower soft tissue absorption, as well as allowing the reduction of bone tissue interference (26). Despite these advantages, previous studies noted that plasma proteins adhere to the surface of bare gold nanoparticles, forming large aggregates which interfere with the pharmacokinetics and biodistribution of the compound (26,29,30).

The rapid clearance and aggregation of AuNPs can be prevented by using a coating agent such as polyethylene glycol (PEG) (25,26,29). The coating can be done using a short-strand PEG derivative, such as PEG-SH or a longer one such as OPSS-PEG-SVA (orthopiryridyldisulfide-polyethylene glycol-N-hydroxysuccinimide). Advantages of using OPSS-PEG-SVA are the ability to bind a specific targeting molecule to the AuNPs, as well as increasing the distance between the bound molecule and the gold particles therefore avoiding stearic hindrances to receptor binding (25,26,29). Incubating AuNPs with OPSS-PEG-SVA allows the particles to be coated with a highly specific ligand, such as 2-deoxy-D-glucose (2-DG), resulting in a targeting agent specific for one cellular group.

Carcinoma cells have been proven to have a higher metabolic rate as well as faster proliferation, which leads to greater demand of glucose. Such metabolic characteristics can be linked to the over-expression of certain glucose transporters, such as Glut-1 or Glut-4, on malignant cell membranes. Glut-1 has been proved to be expressed by almost all cancerous cell types (31,32). The receptor binds and transports D-glucose within the cell, which is further metabolized into D-glucose-6-phosphate and 1,2-diphosphate (31). D-glucose analogues can be transported by Glut-1 or Glut-4 receptors, but cannot be fully metabolized, therefore remaining inside the cells for longer periods before excretion. This property allows analogues such as 2-DG to be used as specific ligands for malignant cells. Positron emission tomography (PET) scans with radioactive fluorodeoxyglucose (FDG) use the glucose pathway described to successfully target cancerous lesions and metastases, which are not always visible through structural imaging techniques. Targeting a metabolic mechanism instead of a cell membrane receptor is one of the most promising developments in cancer research and could eventually lead to better drug delivery systems and overcoming drug-resistance (31,32).

MDA-MB-231 is a breast cancer cell line that is known to overexpress Glucose transporter 1 (Glut-1). Despite their epithelial origin, MDA-MB-231 cells have a claudin-low basal phenotype, being basal cells with mesenchymal features. They are positive to CD 105, present the CD 44+/CD 24− phenotype and are negative for Ep-CAM and E-cadherin staining.
Together with BT549 and SUM1315, it represents a subtype of basal breast cancer cells with the immunoprofile ER\(^{-}\), PR\(^{-}\), HER2\(^{-}\), with low Ki67, claudin-3, claudin-4 and claudin-7. These types of cancer cells have a high risk of metastasis and an intermediate response to chemotherapy (32-35).

Metastasis can be explained using the “seed and soil” theory, which suggests that cancer cells, which act as seeds, undergo epithelial to mesenchymal transformation (EMT), become circulating tumor cells (CTCs), migrate and eventually infiltrate distant sites as disseminating tumor cells (DTCs). The environmental elements which contribute to these processes and are essential to making distant sites permeable to CTCs and DTCs are viewed as the “soil”. One of the most important parts of the “soil” is the immune system. Tumor associated macrophages (TAMs) represent a category of macrophages recruited by tumor cells which are subjected to immunosuppressive stimuli such as IL-10 and are therefore similar to M2 activated macrophages. However, instead of performing defensive tasks, they take on a trophic and immunosuppressive role. Moreover, TAMs can occupy up to 40% of the mass of a solid tumor and have been previously associated with a poor case prognosis (36). These characteristics have raised an interest in the potential use of macrophages in cancer imaging and treatment (37,38).

Previous studies used the macrophage cell line RAW 264.7 to create an in vitro model that would simulate the interactions between malignant cells and TAMs (39). RAW 264.7 cells overexpress Glut-1 transporters and therefore exhibit increased glucose uptake and metabolism (40).

This study focused on developing a targeted radiocontrast agent based on gold nanoparticles that would allow cancer detection. The imaging platform consisted of 2-deoxy-D-glucose covalently coupled to spherical gold nanoparticles to target Glut-1 over-expression in breast cancer cells. The feasibility of this platform was examined using CT imaging and histological staining. The team synthesized long circulating gold nanoparticles and focused on demonstrating \textit{in vitro} that the attenuation coefficient for the 2-DG targeted cells is significantly higher than that of non-targeted or non-cancerous cells.

One of the advantages of the proposed imaging technique is the use of a novel targeting agent (AuNPs) which provides superior X-ray attenuation over conventional iodine-based contrast agents. Moreover, it provides the specific targeting of cancer cells with a ligand against the Glut-1 overexpressed by breast cancer cells and macrophages. CT imaging offers excellent tissue penetration and spatial resolution as well as rapid image acquisition.

It was hypothesized that the attachment of 2-deoxy-D-glucose to gold nanoparticles would facilitate the targeting of Glut-1 receptors on the surface of breast cancer cells, MDA-MB-231, and macrophage cells, RAW 264.7, allowing sufficient X-ray attenuation for non-invasive CT imaging.
Materials and Methods

Materials

Sodium citrate, gold chloride and Picrosirius Red stain (Direct Red 80, Picric acid solution and Hematoxylin Solution A according to Weigert) were purchased from Sigma-Aldrich (St. Louis, MO, USA). OPSS-PEG-SVA was purchased from Laysan Bio (Arab, AL, USA). PES membranes (3000 MWCO) were purchased from Fisher Scientific. Silver enhancement staining kit was purchased from Structure Probe, Inc. (West Chester, PA, USA). DMEM, Fibroblast Basal Media and Fibroblast Serum-Free Growth kit were purchased from ATCC (Manassas, VA, USA). Primary anti-Glut1 antibody was purchased from Fisher Scientific, Alexa Fluor 288 goat anti-rabbit IgG (H+L) was purchased from AbCam (Cambridge, MA, USA), Vectashield mounting medium with DAPI was purchased from Vector Laboratories Inc. (Burlingame, CA, USA), Phalloidin was purchased from Invitrogen (Carlsbad, CA, USA), MTT assay kit was purchased from Roche Applied Science (Indianapolis, IN, USA), 2-Deoxy-D-Glucose was purchased from VWR, D (+)-Glucosamine hydrochloride was purchased from Fisher Scientific.

Gold Nanoparticle Synthesis

The gold nanoparticles were prepared in flasks previously cleaned with nitric acid. The base for the solution consisted of 500 mL of nanopure water which was filtered through a 0.22 µm filter and boiled in a 1 L conical flask. 5 mL of Gold Chloride (10%) was added to the boiling water followed by 4mL of Sodium Citrate solution (1%). As the compounds were added, the solution changed in color from transparent to yellow, grey, black and finally resulting in a dark burgundy liquid. The solution was left to boil until 200-250 mL remained. After cooling down, the AuNPs were PEGylated with a polyethylene glycol derivative to avoid aggregation in vivo. The AuNPs were incubated overnight with 10 mg of orthopyridyl-disulfide-polyethylene-glycol-N-hydroxysuccinimide (OPSS-PEG-SVA) for the covalent coupling of the peptide. The adequate proportion between PEG and the gold substrate can be determined using a flocculation assay. A previous study on AuNPs and their applications in cardiovascular imaging found that the minimum amount of PEG required to stabilize the AuNPs was a ratio of 100:1 PEG: AuNPs (20). Following PEGylation the AuNPs were concentrated by centrifugation at 3270 rpm for 1 hour. The light purple supernatant was discarded and the dark solution of AuNPs was further concentrated using PES membrane filter tubes (3000 MWCO).

Characterization of Gold Nanoparticles

The AuNPs were characterized in terms of size and polydispersity by UV Spectrophotometry and Dynamic Light Scattering (Malvern Nano-ZS Zetasizer, Malvern Instruments Ltd., a Spectris Company; Worcestershire, UK). The particles were analyzed using an Atomic Force Microscope.
Synthesis of 2-deoxy-D-glucose-AuNPs

Two different methods were used and compared for the synthesis of 2-deoxy-D-glucose-AuNPs. For the first method, the AuNP solution was centrifuged through 3000 molecular weight membranes to achieve a concentration of 6 nM. A solution of 2-deoxy-D-glucose (2-DG) was made by adding 4 mg of 2-DG to 2 mL of nanopure water. The solution was then combined with 2.2 mL of AuNPs solution and left to stir overnight at room temperature. The solution was further centrifuged through PES membrane tubes at 3270 rpm, for 1 hour. Following centrifugation, the unbound 2-DG solution was seen at the bottom of the tubes in the form of a clear liquid. The 2-DG-AuNP solution remained above the filter, due to the larger molecular size. It appeared as a very concentrated black solution. The 2-DG-AuNPs were reconstituted with 1 mL of nanopure water (Method 1). For the second method, the same protocol was followed, but a solution of 2-amino-deoxy-D-glucose (2-NH₂-DG) was used instead of 2-DG, resulting in 2-NH₂-DG-AuNPs (Method 2).

Assessment of Glut-1 Expression in cells

The cells were grown in DMEM medium supplemented with 10% FBS and antibiotics at 37°C in 5% CO₂, in a 96-well plate. The next day, the cells were washed with 100 µl of PBS and fixed with 40 µl of formalin for 20 minutes at room temperature. After washing again with 100 µl of PBS, they were incubated with 100 µl of 2% BSA and 0.1% Tween 20 for 1 hour and then incubated with 100 µl of anti-Glut1 antibody, at 1: 1,000 dilution in PBS, v/v, for 2 hours at 25°C. The unbound anti-Glut1 antibody was removed from the cells by washing three to five times with PBS and the cells were incubated with 100 µl of the secondary antibody, goat anti-rabbit IgG Alexa–Fluor 288, at 1: 1,000 dilution in PBS, v/v, for 1 hour at room temperature. The excess secondary antibody was washed away three to five times with PBS and then 100 µl of ABTS substrate was added. The fluorescence at 488 nm/520 nm was measured with a plate reader.

Immunocytochemistry

The cells were grown overnight on 8-chamber slides. They were fixed with 300 µl of 4% formalin for 10 minutes and then incubated with 500 µl of 1% BSA (10% normal goat serum, 0.3M glycine) in 0.1% PBS-Tween for 1 hour to permeabilize the cells and block non-specific protein-protein interactions. The cells were then incubated with 300 µl of antibody (ab652, 1:1000 dilution) overnight at 4°C. The secondary antibody (green) was Alexa Fluor® 288 goat anti-rabbit IgG (H+L) used at a 1:1000 dilution for 1 hour. DAPI (mounting media) was used to stain the cell nuclei (blue) at a concentration of 1.43 µM. Phalloidin (red) was used for staining actin filaments. Fluorescence microscopy was used to determine the Glut-1 expression in cells.
Internalization of AuNPs in cells

The cells were grown overnight on 8-chamber slides. 50 µl of AuNP solution was added directly into the media and the cells were incubated for 2, 4 and 24 hours at 37 °C and 5% CO₂. The cells were then washed three times with warmed PBS and fixed with 300 µl of 4% formaldehyde for 20 minutes at room temperature. The cells were washed three times with PBS and then incubated with 100 µl of Hematoxylin for 15 minutes to stain the nuclei. After the cells were washed again, they were stained for AuNPs with 100 µl of silver staining for 12 minutes. The slides were then washed and dried followed by mounting. Light microscopy was performed to determine the distribution of AuNPs retention in the cells.

Cell Culture

Human breast carcinoma cell line MDA-MB-231, human macrophage cell line RAW 264.7 and human fibroblast cell line MC 3T3.E1 were used for all the experiments. Human umbilical vein endothelial cells (HUVEC) were used for part of the experiments. The MDA-MB-231 cells and RAW 264.7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and antibiotics, and incubated at 37 °C in a humidified incubator with an atmosphere of 5% CO₂: 95% air. The HUVEC cells were grown in Vascular Cell Basal Medium with Endothelial Cell Growth Kit VEGF (rh VEGF: 5 ng/mL, rh EGF: 5 ng/mL, rh FGF basic: 5 ng/mL, rh IGF-1: 15 ng/mL, L-glutamine: 10 mM, Heparin sulfate: 0.75 Units/mL, Hydrocortisone: 1 µg/mL, Ascorbic acid: 50 µg/mL, Fetal bovine serum: 2%) and antibiotics. Fibroblasts (MC 3T3.E1 cell line) were grown in Fibroblast Basal Media with Fibroblast Serum-Free Growth kit (HLL Supplement: HSA 500 µg/mL, linoleic acid 0.6 mM, lecithin 0.6 µg/mL, L-Glutamine: 7.5 mM, rh FGF basic: 5 ng/mL, rh EGF/TGF-1 Supplements: 5 ng/mL and 30 pg/mL, respectively, rh Insulin: 5 µg/mL, Hydrocortisone: 1 µg/mL, Ascorbic acid: 50 µg/mL). Cell viability was measured before every experiment using Trypan blue, which stains dead tissues or cells. The cells were counted and analyzed using the Vi-CELL™ XR Cell Counter.

Computed Tomography Scans

One milliliter of cell suspension (10^5 cells/mL) was mixed with 1 mL of non-targeted AuNPs and allowed to interact for 4 hours at 37°C. The same procedure was repeated for 2-DG-AuNPs, 2-DG-amino-AuNPs and PBS (control). Then the solutions were centrifuged 3 times at 1000 rpm for 5 minutes, to wash out unbound AuNPs. After each centrifugation step the mixture was resuspended in PBS solution (1 mL total volume). The cell suspensions were then analyzed with computed tomography. CT imaging was performed with a GE Ultra flat panel CT scanner (General Electric, Milwaukee, WI) with the following acquisition settings: 80kVp, 22 mA with 16 s rotation/exposure. Simple back projections were obtained for the 0.154 µm image reconstruction and exported as DICOM images. Image analysis was performed using the OsiriX software.
Cell Viability Assay

The AuNPs were tested for cytotoxicity using the MDA-MB-231 and RAW 264.7 cell lines. The cells were incubated on a 96-well plate with 50 µl of AuNPs, 2-DG-AuNPs and PBS (control) for 4 hours. The cells were washed 3 times with PBS and then 100 µl of DMEM and 10 µl of MTT reagent were added to the wells. After incubating for 2 hours, the cell viability was measured using a plate reader. In this assay, cells that properly metabolize a dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) undergo visible color changes, while cells that are incapable of metabolizing the dye remain colorless. The color changes were assessed spectrophotometrically at 488/520 nm.
Results

Gold Nanoparticle Synthesis and Characterization

The gold nanoparticles were synthesized by citrate reduction, using the Turkevich method. All the AuNP preparations used in this study were analyzed using UV spectrometry, DLS and atomic force microscopy (AFM). For all the samples, the UV absorbance peaked at 540 nm, with 2-DG-AuNPs (Method 1) showing the highest absorbance. The AFM analysis showed a uniform preparation of AuNPs of 40-50 nm, at 2.0 µm amplitude (Figure 1).

![Figure 1. Characterization of gold nanoparticles: UV Absorption of PEG-stabilized AuNPs (bare AuNPs, 2-DG-AuNPs Method 1 and Method 2) and AFM image of AuNPs](image)

The bare AuNP samples had a diameter of 46 nm and 53.8 nm, respectively, and a polydispersity index (PDI) value of 0.451 and 0.576. Method 1 and Method 2 AuNPs had a diameter of 42.2 nm and 43 nm, with a PDI value of 0.374 and 0.405, respectively. All samples registered two peaks, except for Method 1 AuNPs, which only had one peak at 70.6 nm (Table 1, Figure 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diameter (nm)</th>
<th>Polydispersity Index (PDI)</th>
<th>First Peak</th>
<th>Second Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuNPs 7.3 nM</td>
<td>46 nm</td>
<td>0.451</td>
<td>82 nm (87.8%)</td>
<td>9.5 nm (12.2%)</td>
</tr>
<tr>
<td>AuNPs 17.2 nM</td>
<td>53.8 nm</td>
<td>0.576</td>
<td>148 nm (72.4%)</td>
<td>20 nm (27.6%)</td>
</tr>
<tr>
<td>2-DG-AuNPs Method 1</td>
<td>42.2 nm</td>
<td>0.374</td>
<td>70.6 nm (100%)</td>
<td>N/A</td>
</tr>
<tr>
<td>2-NH₂-DG-AuNPs Method 2</td>
<td>43 nm</td>
<td>0.405</td>
<td>66.3 nm (86.4%)</td>
<td>8.44 nm (13.6%)</td>
</tr>
</tbody>
</table>

Table 1. DLS Analysis of PEG-stabilized AuNPs
Assessment of Glut-1 Expression in Cells

Glut-1 expression was assessed using an ELISA assay, measured at excitation 488 and emission 520. MDA-MB-231 cells displayed a higher Glut-1 concentration, 131.33 absorbance, when compared to HUVEC cells, 120.50 absorbance. The difference between the MDA-MB-293 cells incubated with Glut-1 antibody (131.33 absorbance) and the control MDA-MB-293 cells (125.00 absorbance) was statistically significant in the three ELISA assays conducted (paired t-test, p<0.05). The difference between HUVEC cells and MDA-
MB-293 was not statistically significant based on the results from two ELISA assays (non-paired t-test, p>0.05).

![Bar chart showing Glut-1 expression levels in different cell types.](image)

**Figure 3.** Assessment of Glut-1 expression in breast cancer cells, MDA-MB-231, and human umbilical vein endothelial cells, HUVEC.

**Immunocytochemistry**

Glut-1 expression was also assessed using immunocytochemistry by staining Glut-1 receptors with green Alexa Fluor® 288 goat anti-rabbit IgG (H+L). Breast cancer cells MDA-MB-231 (image B, Figure 4) showed the highest Glut-1 receptor expression, when compared to macrophage cells RAW 264.7 (image A, Figure 4), and fibroblast control cells MC 3T3.E1 (image C, Figure 4). Macrophage cells showed some Glut-1 receptor expression (image A, Figure 4), while fibroblasts did not display any Glut-1 expression.

![Images of fluorescence microscopy for Glut-1 expression in different cell types.](images)

**Figure 4.** Fluorescence microscopy images of Glut-1 expression in macrophage cells (image A), breast cancer cells (image B) and fibroblasts (image C); scale bar represents 100 µm.
Internalization of AuNPs in Cells

Gold nanoparticle uptake was analyzed after incubation with a AuNPs solution for four hours using light microscopy. The gold nanoparticles were stained with silver and appear as small black particles on the images (Figure 5 and Figure 6). Both macrophage cells RAW 264.7 and breast cancer cells MDA-MB-231 displayed AuNPs internalization compared to fibroblast cells MC 3T3.E1 (Figure 5 and Figure 6). Macrophages showed the most infiltration with the Method 1 preparation, which can be noted because of the presence of darker aggregates inside the cells (image B, Figure 5). Fibroblasts showed no difference in gold nanoparticle uptake between the AuNPs, Method 1 and control (images D-F, Figure 5).

Breast cancer cells MDA-MB-231 showed the highest amount of internalization for Method 1 nanoparticles, but also displayed some infiltration for the bare AuNPs solution and Method 2 solution (Figure 6).
Computed Tomography Scans

Breast cancer cells MDA-MB-231, macrophages RAW 264.7 and fibroblasts MC 3T3.E1 were scanned using a pre-clinical CT to analyze gold nanoparticle uptake. Samples that display a higher uptake signal appear red on the CT images. Breast cancer cells showed the most intense signal for Method 1 2-DG-AuNPs at 916 HU, and Method 2 2-NH$_2$-DG-AuNPs at 435 HU, with almost no signal registered for the bare AuNPs, at 22 HU as opposed to 19 HU for the control sample (Table 2). No signal was visible on the scan in the control and bare AuNPs sample (Figure 7).
For macrophages, the Method 1 sample also displayed the highest radiointensity (767 HU), followed by bare AuNPs (140 HU). Control samples registered 20 HU for fibroblast cells and 25 for macrophages (Table 2). A small radiointense signal was visible on the scan for bare AuNPs in fibroblast cells (Figure 8).
Figure 8. Computed Tomography images of AuNPs internalization in macrophage RAW 264.7 cells and fibroblast MC 3T3.E1 cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control</th>
<th>AuNPs</th>
<th>Method 2</th>
<th>Method 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>19 HU</td>
<td>22 HU</td>
<td>435 HU</td>
<td>916 HU</td>
</tr>
<tr>
<td>RAW 264.7</td>
<td>25 HU</td>
<td>140 HU</td>
<td>NA</td>
<td>767 HU</td>
</tr>
<tr>
<td>MC 3T3.E1</td>
<td>20 HU</td>
<td>23 HU</td>
<td>NA</td>
<td>32 HU</td>
</tr>
</tbody>
</table>

Table 2. Radiointensity of three cell lines after treatment with gold nanoparticles.

**Cell Viability**

Following incubation for 4 hours with gold nanoparticles, cell viability was measured for the three cell lines used in the study: MDA-MB-231, RAW 264.7 and MC 3T3.E1. Absorbance values higher than the control values indicate an increase in proliferation rate. The MTT assay showed a more intense purple coloration in the Method 1 and Method 2 samples. The cells treated with gold nanoparticles were compared to cells treated with PBS, as well as gold nanoparticle solution without MTT reagent. None of the samples showed a lower absorbance value than the control, therefore showing no decline in the cell proliferation rate when treated with gold nanoparticles.
Figure 9. MTT Viability Assay for gold nanoparticle incubation using three cell lines: fibroblast MC 3T3.E1 cells, breast cancer MDA-MB-231 cells, macrophage RAW 264.7 cells.
Discussion

Medical imaging techniques can be divided into structural and functional imaging. Recently, functional imaging has been gaining interest over more conventional anatomical imaging. This came as a response to the need for earlier detection of malignant tissues and metastases, which were not visible through structural scans. This study proposed a novel approach to functional imaging, using gold nanoparticles to target a ligand specific to mesenchymal breast cancer cells and macrophages.

Gold nanoparticles were synthetized by citrate reduction and all the batches were analyzed by UV spectrophotometry and Dynamic Light Scattering (DLS), checking particle size and dispersity to insure the accuracy of the experiments. Four preparations were necessary to conclude the experimental tests, consisting of bare AuNPs (no ligand attached), 2-NH₂-DG-AuNPs (Method 2) and two batches of 2-DG-AuNPs (Method 1), which were eventually combined. Bare AuNPs were synthesized at two different concentrations, 7.3 nM and 17.2 nM (Table 1 and Figure 2). The protocol that was chosen for AuNP synthesis was meant to result in a homogenous solution of nanoparticles with a diameter ranging from 40 to 60 nm. This size was hypothesized to be small enough to infiltrate cancerous tissue, but also large enough to prevent rapid clearance and excretion by the reticuloendothelial system. Based on the results from UV spectrophotometry, atomic force microscopy (AFM) and DLS, the gold nanoparticles used in this study were 42.2-53.8 nm in size, which was well within the proposed range (Figure 1 and Table 1). The samples were relatively homogenous, displaying polydispersity index values between 0.374 and 0.576 (Table 1).

Colloidal gold has been found to be unstable in a saline environment. The AuNPs were stabilized using a functionalized long chain PEG with a molecular weight of 5 kDa (OPSS-PEG-SVA) to prevent the formation of aggregates and rapid clearance in vivo. Moreover, OPSS-PEG-SVA had functional groups available for covalent bonding which allowed the conjugation via strong gold-thiolate bonds of a specific ligand (a D-glucose analogue). Two different ligands were tested, 2-DG (Method 1) and 2-NH₂-DG (Method 2).

The proposed imaging platform was based on the overexpression of glucose transporters (Glut-1) on the cell membranes of mesenchymal breast cancer cells MDA-MB-231 and macrophages RAW 264.7. Glut-1 expression was first determined using an ELISA assay, measured at 488 excitation and 520 emission. HUVEC cells were used as control group in this experiment and showed a significantly lower expression of Glut-1 compared to MDA-MB-231 (Figure 3). Although the difference between HUVEC and MDA-MB-231 was statistically significant based on the three ELISA assays conducted, HUVEC cells did express some Glut-1 and were not considered representative for the study. The cell line was therefore terminated following these experiments.

To verify the presence of Glut-1 on cancer cells and macrophages, the cells were imaged using immunocytochemistry. Following staining with Glut-1 antibody (green), MDA-MB-231 cells showed the highest Glut-1 expression (most intense green coloration), followed by RAW 264.7. Fibroblasts MC 3T3.E1 were used as a control group and displayed no Glut-1 expression (Figure 4). These results confirmed the premises of the hypothesis and therefore allowed the study to continue to the in vitro AuNP experimental phase. A pilot was first conducted to determine the adequate incubation time with gold nanoparticles. Cells were incubated with AuNPs for 2, 4 and 24 hours and the results were analyzed histologically. It was concluded that 2 hours did not allow sufficient gold internalization, while 24 hours allowed too much time for the clearance of the nanoparticles. Therefore the 4-hour time point was used in the following experiments.
The AuNP internalization was first observed histologically, using a silver compound to stain the nanoparticles. Silver staining gave AuNPs a dark, almost black coloration. Therefore, when comparing MDA-MB-231 and RAW 264.7 slides to MC 3T3.E1, a darker coloration of the Glut-1 expressing cells can be noted, due to small dark aggregates present inside said cells. Moreover, MDA-MB-231 and RAW 264.7 cells which were incubated with PBS confirm these results, displaying a light coloration, with no dark particles visible (Figure 5 and Figure 6). When comparing bare AuNPs and functionalized 2-DG-AuNPs, a slight difference could be noted in MDA-MB-231 cells, which displayed higher internalization for functionalized nanoparticles (Figure 6). However, for RAW 264.7 cells, the amount of internalization for bare AuNPs and 2-DG-AuNPs was similar (Figure 5). A reason for this could be the presence of other mechanisms (independent of glucose transporters) for internalization in macrophage cells. For the experiments using breast cancer cells MDA-MB-231, both Method 1, 2-DG-AuNPs, and Method 2, 2-NH₂-DG-AuNPs were used. Better cell infiltration was observed for the Method 1 preparation and, therefore, the following experiments were conducted without Method 2 AuNPs.

The designed targeting agents, 2-DG-AuNPs and 2-NH₂-DG-AuNPs, were tested using a pre-clinical CT. Gold nanoparticles were more radiointense than air (black) or PBS (green) and therefore appeared red on the CT scans. As the cells were washed and centrifuged before scanning, the cells gathered at the bottom of the sample tubes, forming cell pellets. The radiointensity measurements were done in triplicates using values from the bottom of the sample tubes, representative for each cell group. It could be noted that Method 1 AuNPs displayed the highest radiointensity both when measured with the OsiriX software and visually on the scans (Figure 7, Figure 8 and Table 2). 2-DG-AuNPs registered values of 916 HU for MDA-MB-231 cells and 767 HU for RAW 264.7 cells (Table 2). Method 2 AuNPs also displayed a relatively high radiointensity of 435 HU for MDA-MB-231 (Figure 7 and Table 2).

A surprising result was the presence of a light radiointense signal in the MC 3T3.E1 sample incubated with 2-DG-AuNPs (Figure 8). Considering the results of the histological staining, where no AuNPs internalization was noted in fibroblast cells, it could be hypothesized that this result was due to binding of gold nanoparticles on the exterior of the cells or the cell pellet. Despite the intense red signal present on the CT image, when measured, the radiointensity was a lot lower than the values displayed by Glut-1 overexpressing cells, being 32 HU as opposed to 767 HU or 916 HU (Table 2). Even though the results for 2-DG-AuNPs testing confirm the study hypothesis, the results registered for non-functionalized AuNPs could offer more insight into the mechanisms responsible for gold nanoparticle internalization and excretion.

AuNPs seemed to be internalized as much as 2-DG-AuNPs on the histology slides, with a slight difference in the MDA-MB-231 cells (Figure 6 and Figure 6). However, CT imaging showed almost no radiointensity for the cells treated with bare AuNPs (Figure 7 and Figure 8). This could indicate that AuNPs were not internalized through the same mechanism as 2-DG-AuNPs and that they could be eliminated at a faster rate by the cells upon washing and centrifugation. The only cell group that displayed a radiointense signal for non-functionalized AuNPs were macrophages (Figure 8). Since macrophage cells are phagocytes it could be assumed that this was the mechanism used to internalize AuNPs, independently of the glucose pathway that was targeted.

To insure safe applications in vivo, cell toxicity was assessed for the compounds used in the study. The MTT reagent used to test viability was metabolized by live cells, gaining an intense purple color. When measuring the light absorbance with a plate reader, a higher value than the control value indicated an increase in proliferation rate. Bare AuNPs exhibited similar absorption compared to control samples, treated with PBS (Figure 9). However, cells
treated with Method 1 or Method 2 AuNPs showed a more intense purple coloration than the control cells, which could indicate an increase in the proliferation rate. The team hypothesized that this coloration appeared due to the purple color of the AuNPs solution and therefore measured the absorption for colloidal gold nanoparticles without MTT reagent. AuNPs solution displayed an absorption value of only 0.05, concluding that it did not affect the results of the assay. Further tests are needed to determine what caused the increased absorption for the cells treated with functionalized AuNPs, but it can be concluded that treatment with gold nanoparticles does not affect cell viability negatively.

Limitations and Possibilities

Although the results confirmed the hypothesis, a limitation of the study was the inability to systematically quantify and statistically analyze some of the experiments. Histological and radiological results were reproduced objectively, but they could not be quantified (histology) and analyzed statistically. Even though numerical values were provided for the radiointensity of samples on CT scans, the study lacked the time necessary to repeat the experiments and to gain enough power for accurate statistical testing.
Conclusion

This study was the first attempt at using gold nanoparticles functionalized with 2-deoxy-D-glucose to trace and image breast cancer and tumor-associated macrophages. Functionalized 2-DG-AuNPs proved to be a valid targeting contrast agent for cell lines MDA-MB-231 and RAW 264.7, exhibiting high radiointensity upon CT imaging and low toxicity. Gold internalization was verified with histological and radiological techniques and the positive results indicate a need for further research into this topic. The targeting agent developed, 2-DG-AuNPs, promises to be a cheap and safe alternative for other types of functional imaging techniques such as nuclear imaging with radioactive fluorodeoxyglucose (FDG).
Bibliography


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