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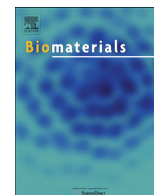

Bisphosphonate-functionalized gold nanoparticles for contrast-enhanced X-ray detection of breast microcalcifications

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ABSTRACT

Microcalcifications are one of the most common abnormalities detected by mammography for the diagnosis of breast cancer. However, the detection of microcalcifications and correct diagnosis of breast cancer are limited by the sensitivity and specificity of mammography. Therefore, the objective of this study was to investigate the potential of bisphosphonate-functionalized gold nanoparticles (BP-Au NPs) for contrast-enhanced radiographic detection of breast microcalcifications using two models of breast microcalcifications, which allowed for precise control over levels of hydroxyapatite (HA) mineral within a low attenuating matrix. First, an *in vitro* imaging phantom was prepared with varying concentrations of HA uniformly dispersed in an agarose hydrogel. The X-ray attenuation of HA-agarose compositions labeled by BP-Au NPs was increased by up to 26 HU compared to unlabeled compositions for HA concentrations ranging from 1 to 10 mg/mL. Second, an *ex vivo* tissue model was developed to more closely mimic the heterogeneity of breast tissue by injecting varying concentrations of HA in a Matrigel carrier into murine mammary glands. The X-ray attenuation of HA-Matrigel compositions labeled by BP-Au NPs was increased by up to 289 HU compared to unlabeled compositions for HA concentrations ranging from 0.5 to 25 mg/mL, which included an HA concentration (0.5 mg/mL) that was otherwise undetectable by micro-computed tomography. Cumulatively, both models demonstrated the ability of BP-Au NPs to enhance contrast for radiographic detection of microcalcifications, including at a clinically-relevant imaging resolution. Therefore, BP-Au NPs may have potential to improve clinical detection of breast microcalcifications by mammography.

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1. Introduction

Breast cancer is the most common cancer and second leading cause of cancer-related deaths among women in the United States [1]. Early detection by mammography is the current clinical gold standard in breast cancer screening and is considered to be the best hope for controlling the disease due to a demonstrated decrease in breast cancer mortality [2,3]. Microcalcifications are one of the most common abnormalities detected by mammography [4,5] and are coincident with 30–50% of breast cancers detected by mammograms [6–8]. Microcalcifications are deposits of hydroxyapatite (HA) or calcium oxalate (CaOx) mineral within the breast tissue [9–12], which exhibit high X-ray attenuation relative to the low attenuating breast tissue facilitating detection by mammography.

The detection of microcalcifications and correct diagnosis of breast cancer are limited by the sensitivity and specificity of mammography. Mammographically-detected microcalcifications range in size from 0.1 to 1.0 mm, but smaller microcalcifications are detected histologically [6]. Limitations in sensitivity can result in the missed detection of cancerous lesions at an early stage, or false negatives, and therefore lost opportunity for early treatment [13]. Studies have reported that 10–30% of all breast lesions are missed during routine mammographic screening [6,14]. Limitations in specificity can result in false positives [15] and unnecessary biopsies, which increase patient anxiety and healthcare costs [16]. The detection of microcalcifications is limited by their small size and the presence of other structures in the breast, such as fibrous tissue, ducts and blood vessels, which may be mistaken for microcalcifications due to exhibiting greater X-ray attenuation than surrounding tissue [6]. Therefore, improvements are needed for

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detecting microcalcifications during mammographic screening for breast cancer.

X-ray contrast agents [17] could be used to improve the sensitivity and specificity of detecting breast microcalcifications by mammography. Improved sensitivity could be achieved by providing enhanced X-ray contrast for small microcalcifications that are otherwise below the detection limit of mammography, thus enabling earlier detection. Improved specificity could be achieved using a targeted X-ray contrast agent.

Gold nanoparticles (Au NPs) have received recent attention as a potential X-ray contrast agent due to exhibiting greater X-ray attenuation and improved vascular retention compared to iodinated molecular agents [18–20]. Additionally, Au NPs are widely reported to be non-cytotoxic, readily synthesized, and readily surface functionalized for colloidal stability and targeted delivery [21]. Au NPs surface functionalized with ligands such as antibodies [22–25], peptides [26] or other tumor-specific biomarkers [27] have demonstrated potential as an actively targeted X-ray contrast agent for cancer cells *in vitro* or tumors *in vivo*.

Bisphosphonate-functionalized Au NPs (BP-Au NPs) were recently investigated as a targeted X-ray contrast agent for microdamage in bone tissue [28,29]. Bisphosphonates, such as alendronate, are used as a pharmaceutical in the treatment of osteoporosis [30] due to exhibiting a high binding affinity for HA, the mineral component of bone [31]. BP-Au NPs exhibited colloidal stability in physiological media, a high binding affinity for HA crystals *in vitro*, and targeted labeling of damaged bone tissue *in vitro* [28,29]. Interestingly, HA is also the mineral component of the type of breast microcalcifications known to be associated with malignant lesions [9–12,32].

Therefore, the objective of this study was to investigate the potential of BP-Au NPs for contrast-enhanced radiographic detection of breast microcalcifications. Two models of breast microcalcifications were developed for precisely controlling levels of HA mineral within a low attenuating matrix. First, an *in vitro* imaging phantom was prepared with varying concentrations of HA uniformly dispersed in an agarose hydrogel. Second, an *ex vivo* murine tissue model was developed to more closely mimic the heterogeneity of breast tissue, and also lay the groundwork for an *in vivo* model, by injecting varying concentrations of HA in a Matrigel carrier into the fat pad of the number 4 mouse mammary gland.

2. Materials and methods

2.1. Synthesis and characterization of BP-Au NPs

Au NPs were synthesized to a mean particle diameter of ~13 nm using the citrate reduction method [33], as previously described in detail [28,29]. Briefly, 0.1 g gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$, ≥99.9%, Sigma–Aldrich, St. Louis, MO) was added to 500 mL de-ionized (DI) water and brought to a boil while stirring. Once boiling, 0.5 g of trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$, ACS reagent, >99%, Sigma–Aldrich) was added to the gold solution and left to boil, while covered, for 20 min before removing heat and stirring overnight. The solution volume was adjusted back to 500 mL after reaching room temperature, resulting in a final solution with a gold concentration of ~0.5 mM and a red wine color.

Au NPs were prepared for bisphosphonate surface functionalization by mixing 240 mL of as-synthesized Au NPs with 10 mL 2 wt% polyvinyl alcohol (PVA 10-98, 61,000 Da, Fluka, St. Louis, MO) and 5.8 g ion exchange resin (Dowex Marathon, MR-3, Sigma–Aldrich, St. Louis, MO) to remove excess ions. The spent resin was then filtered from the Au NPs (grade 3, Whatman, Piscataway, NJ). Au NPs were surface functionalized with alendronate sodium trihydrate ($C_4H_{12}NaNO_7P_2 \cdot 3H_2O$, ≥97%, Sigma–Aldrich, St. Louis, MO), which provided a primary amine for binding gold opposite a bisphosphonate functional group for targeting calcium in HA. Four mL of a 0.01 M solution of alendronate was added to 250 mL of PVA-Au NPs and left to stir overnight. Excess bisphosphonate molecules were removed by dialysis (Spectra/Por, MWCO = 3500 Da, Spectrum Laboratories, Rancho Dominquez, CA) against DI water for 3 d, changing the water at least 3 times daily.

BP-Au NPs have been thoroughly characterized in previous studies, which included measurements of the mean particle diameter, mean hydrodynamic diameter, colloidal stability in physiological media, and bisphosphonate surface density [28,29]. All BP-Au NPs prepared in this study were characterized by dynamic light scattering (DLS, Zetasizer Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK) and ultraviolet-visible (UV–Vis) spectroscopy (Nanodrop 200C, Thermo Scientific, Wilmington, DE) to ensure consistency in the mean hydrodynamic diameter and colloidal stability, respectively. The hydrodynamic diameter was measured using DLS after diluting as-prepared BP-Au NPs in DI water to a final gold concentration of ~0.5 μM and reported as the mean of three samples. Colloidal stability was verified by measuring the surface plasmon resonance (SPR) peak using UV–Vis spectroscopy after diluting as-prepared BP-Au NPs in DI water to a final gold concentration of 0.4 mM. Gold concentrations were measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 3000, Perkin Elmer, Inc. Waltham, MA) after acidifying with 3% aqua regia (3 HCl:1 HNO_3). Calibration curves were created by diluting certified standard gold solutions (Assurance Grade, SPEX CertiPrep, Metuchen, NJ).

2.2. In vitro imaging phantom

An *in vitro* imaging phantom of breast microcalcifications was created by mixing varying concentrations of HA crystals in an agarose hydrogel. Whisker-shaped HA crystals, exhibiting a mean length and width of ~18 x ~2 μm, were synthesized using the chelate decomposition method, as described elsewhere [34]. The specific surface area of the HA crystals was 5.63 m^2/g as measured using Brunauer-Emmett-Teller (BET) N_2 adsorption (Autosorb-1, Quantachrome Instruments, Boynton Beach, FL) [28]. HA crystals were added to a 2 wt% agarose hydrogel (Sigma–Aldrich, St. Louis, MO) at varying HA concentrations of 0, 0.5, 1.0, 2.5, 5.0, 10, and 25 mg/mL and gelled into 10 μL pellets at 4°C. The HA concentration in each pellet was verified by measuring the calcium concentration with ICP-OES using the methods described above. Calibration curves were created by diluting certified standard calcium solutions (Assurance Grade, SPEX CertiPrep).

HA-agarose pellets labeled by BP-Au NPs were prepared by labeling HA crystals with BP-Au NPs prior to forming the pellets. HA concentrations of 0.5, 1.0, 2.5, 5.0, 10, and 25 mg/mL were incubated in DI water containing 40 mg/L (0.2 mM) BP-Au NPs for 24 h to allow for maximum binding of the BP-Au NPs to the HA crystals [28]. Thus, each HA concentration was exposed to the same BP-Au NP concentration for the same length of time. Unbound BP-Au NPs were separated from HA crystals by centrifugation at ~700 g for 2 min. HA crystals labeled by BP-Au NPs were collected using 0.45 μm filter paper (Nylaflo, Pall Corporation, Ann Arbor, MI), rinsed with 10 mL DI water, dried overnight in an oven at 37°C, and stored under vacuum. Labeled HA crystals were mixed with 2 wt% agarose solution and gelled into 10 μL pellets at 4°C.

Labeled and unlabeled HA-agarose pellets were imaged in Eppendorf tubes by micro-computed tomography (micro-CT, μCT-80, Scanco Medical AG, Brüttisellen, Switzerland) at 45 kVp, 177 μA, 600 ms integration time, and 10 μm resolution for 250 slices. The standard 0.5 mm aluminum filter was removed to increase the number of low energy photons to approximate mammographic imaging. The mean (±standard deviation) sampled volume for each pellet was 5.2 (1.3) mm^3. The measured mean linear attenuation coefficient of the HA-agarose pellets was converted to Hounsfield units (HU) using an internal sample calibration for air (−1000 HU) and water (0 HU) controls measured from 10 slices in the same sample tube. The X-ray attenuation measured for each labeled and unlabeled HA concentration was reported in HU as the mean (±standard deviation) of five samples.

The percent of BP-Au NPs bound to HA crystals was measured as the concentration of unbound BP-Au NPs subtracted from the initial BP-Au NP concentration, divided by the initial BP-Au NP concentration. The initial gold concentration and the concentration of unbound BP-Au NPs were measured using ICP-OES, as described above. The mass of BP-Au NPs bound per HA surface area (mg Au/m^2 HA), or surface density, was calculated using the measured specific surface area of the HA crystals. The mean (±standard deviation) percent of BP-Au NPs bound and mass of BP-Au NPs bound per HA surface area were measured from three samples for each HA concentration. The presence and surface density of BP-Au NPs on HA crystal surfaces were also verified using field emission scanning electron microscopy (FE-SEM, 400 XHR, FEI, Hillsboro, OR). Specimens were prepared by dropping a solution of HA crystals labeled with BP-Au NPs in 90% ethanol on a heated SEM stub and coating with 2.5 nm iridium by sputter deposition. Specimens were imaged at an accelerating voltage of 5 kV and current of 6.3 pA.

2.3. Ex vivo tissue model

Mature mammary glands (MGs) from 8 to 12-week-old nulliparous FVB female mice (Charles River Laboratories International, Inc., Wilmington, MA) were used to develop an *ex vivo* tissue model of microcalcifications. All studies were approved by the Institutional Animal Care and Use Committee at the University of Notre Dame and were conducted in accordance with the guidelines of the U.S. Public Health Service Policy for Humane Care and Use of Laboratory Animals. All efforts were made to minimize suffering of the mice. Mice were euthanized and dissected to expose the left and right number 4 MGs. HA crystals were mixed with Matrigel (BD Matrigel, BD Sciences, Bedford, MA), a hydrogel comprised of extracellular matrix proteins, at HA concentrations of 0, 0.5, 5.0, and 25 mg/mL, and HA-Matrigel compositions were immediately injected into the fat pad of MGs at a dose of 50 μL.

HA-Matrigel compositions labeled with BP-Au NPs were prepared by labeling HA crystals with BP-Au NPs prior to mixing with Matrigel. Three concentrations of

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