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Tunable, biodegradable gold nanoparticles as contrast agents for computed tomography and photoacoustic imaging

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Abstract

Gold nanoparticles (AuNP) have been proposed for many applications in medicine. Although large AuNP (>5.5 nm) are desirable for their longer blood circulation and accumulation in diseased tissues, small AuNP (<5.5 nm) are required for excretion via the kidneys. We present a novel platform where small, excretable AuNP are encapsulated into biodegradable poly di(carboxylatophenoxy)phosphazene (PCPP) nanospheres. These larger nanoparticles (Au-PCPP) can perform their function as contrast agents, then subsequently break down into harmless byproducts and release the AuNP for swift excretion. Homogeneous Au-PCPP were synthesized using a microfluidic device. The size of the Au-PCPP can be controlled by the amount of polyethylene glycol-polylysine (PEG-PLL) block co-polymer in the formulation. Synthesis of Au-PCPP nanoparticles and encapsulation of AuNP in PCPP were evaluated using transmission electron microscopy and their biocompatibility and biodegradability confirmed in vitro. The Au-PCPP nanoparticles were found to produce strong computed tomography contrast. The UV-Vis absorption peak of Au-PCPP can be tuned into the near infrared region via inclusion of varying amounts of AuNP and controlling the nanoparticle size. In vitro and in vivo experiments demonstrated the potential of Au-PCPP as contrast agents for photoacoustic imaging. Therefore, Au-PCPP nanoparticles have high potency as contrast agents for two imaging modalities, as well as being biocompatible and biodegradable, and thus represent a platform with potential for translation into the clinic.

Keywords

Gold nanoparticle; metal-polymer nanoparticle; contrast agent; computed tomography; photoacoustic imaging

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Introduction

Nanoparticle contrast agents have gained increasing interest over the past decade.^{1–5} Nanoparticles have several advantages over small molecule contrast agents. They can carry payloads that have contrast properties unavailable in small molecules, and their size, shape and surface characteristics can be specifically engineered. These features can be used to form nanoparticle contrast agents that have long circulation times, accumulation in disease sites, and provide contrast generation for novel imaging methods. Nanoparticle platforms also allow the formation of multimodal and multifunctional agents for imaging with different modalities, as well as simultaneous imaging and therapeutic applications using theranostic nanoparticles.⁶ Gold nanoparticles (AuNP) have been extensively studied as contrast agents for a range of biomedical imaging techniques, and as therapeutic agents.⁷ AuNP are attractive as materials for use in medicine, since they are inert, biocompatible and can be easily modified with surface capping ligands.^{7–9} Furthermore, AuNP have a number of other characteristics that have encouraged their use in biomedical applications. For example, due to gold's high X-ray attenuation, many AuNP formulations have been developed as contrast agents for computed tomography (CT).^{9–15} CT is one of the most extensively used medical imaging methods, and has the capability of producing images of high spatial and temporal resolution at relatively low cost. Limitations of the current, iodinated small molecule blood pool CT contrast agents, which include short circulation half-lives¹⁶ and potential damage to the kidneys,¹⁷ are motivations for developing newer contrast agents such as AuNP.¹³ Photoacoustic (PA) imaging is an experimental technique that couples the high sensitivity of optical imaging with the depth penetration of ultrasound. Gold nanostructures that absorb near infrared light have found many applications as contrast agents in PA imaging.^{17–20} Other imaging modalities such as fluorescence, surface enhanced Raman spectroscopy (SERS) and optical imaging take advantage of properties of gold nanostructures, such as inherent fluorescence, electromagnetic field enhancement and strong light scattering, respectively.^{7,8} Therapeutic applications of gold nanostructures include drug delivery, nucleic acid delivery, photothermal therapy and radiation therapy.^{7,8} Furthermore, clinical trials have shown no adverse effects of gold nanoparticles for therapeutic applications.²¹ Despite these attractive features, no gold agent has yet been FDA-approved, for reasons including concerns over the excretion of AuNP.

The vast majority of the AuNP developed for applications in nanomedicine have been larger than 5.5 nm.^{7,8} Larger AuNP are desirable because of their longer circulation times and accumulation in disease sites. Long circulating nanoparticles are especially advantageous in the case of blood pool contrast agents.¹³ In addition, for applications that involve the use of near infrared light, the types of AuNP structures that absorb strongly in those wavelengths (rods, shells, etc.) are much larger than 5.5 nm.^{7,8,22–24} However, AuNPs larger than 5.5 nm cannot be excreted by the kidneys²⁵ and data shows that about 20–30% of the injected dose is retained in the liver for months after injection,^{26–28} which would be a concern for FDA-approval and eventual clinical application.²⁹ Furthermore, long-term retention of contrast agents may interfere with subsequent imaging sessions. For nanoparticles to have better translational potential, their size should be less than 5.5 nm in diameter on average to allow them to be excreted rapidly.¹³ Furthermore, in order to avoid renal damage and lower the

burden on kidneys, it may be beneficial for the injected contrast agent dose to be filtered via the kidney gradually, *i.e.* over a period of days, as opposed to minutes as is the case with smaller than 5.5 nm nanoparticles. Therefore, a potentially translatable formulation of long-circulating gold nanoparticles would be larger than 5.5 nm, but would slowly break down into sub-5.5 nm components that could be excreted *via* the urinary system. Such an approach would result in low concentrations of gold nanoparticles reaching the kidneys over an extended time, minimizing potential nephrotoxicity. Agents of such design may prove useful for many of the applications mentioned above.

Others have previously reported biodegradable gold nanoparticle platforms.^{20,30,31} However, these platforms have suffered from issues such as a lack of ability to tune the nanoparticle size, low gold to polymer ratios, the need to use large gold nanoparticles or the use of gold nanoparticles that are not stable in serum when released. In this study we sought to address these issues. We synthesized sub-5.5 nm gold nanoparticles and incorporated them into biodegradable poly-di(carboxylatophenoxy)phosphazene (PCPP) nanospheres to form Au-PCPP nanoparticles. The resulting Au-PCPP would maintain their size and structure in the short-term after injection, allowing for their diagnostic potential to be fulfilled. After this period, the polymer will degrade into harmless byproducts such as phosphate, ammonia, and 4-hydroxybenzoic acid^{32–36} and release the small gold cores for excretion (Figure 1). Our synthesis method allows for control over the size of the Au-PCPP, the amount of AuNP loaded and variation in the surface ligand coating of the AuNP incorporated into the PCPP nanospheres. Varying the payload and nanoparticle size allowed the absorbance to be shifted into the near infrared. We have shown that Au-PCPP are biocompatible and biodegradable *in vitro* and are strong dual imaging contrast agents for CT and PA imaging.

Materials and Methods

Materials

Gold (III) chloride trihydrate, sodium borohydride, poly(bis(4-carboxyphenoxy)phosphazene) disodium salt (PCPP, 1 MDa), L-glutathione reduced, 11-mercaptoundecanoic acid (11-MUA), spermine tetrahydrochloride, and titanium oxide were purchased from Sigma-Aldrich (St. Louis, MO). Methoxy-poly(ethylene glycol)-block-poly(L-lysine hydrochloride) (PEG-PLL, PEG MW 5000, PLL MW 4900) was purchased from Alamanda Polymers (Huntsville, AL). HepG2, J774A.1, Renca, and SVEC4-10 cell lines were purchased from ATCC (Manassas, VA). LIVE/DEAD assay kits were purchased from Life Technologies Invitrogen (Grand Island, NY). MTS assay kits (CellTiter 96) were purchased from Promega (Madison, WI). Polyvinyl chloride plastisol (PVCP) was purchased from M-F Manufacturing Co. (Fort Worth, TX, USA).

Gold nanoparticle synthesis

The gold nanocrystals were synthesized *via* a modification of the method of Turkevich.^{37,38} Gold (III) chloride was reduced by addition of sodium borohydride in small batches, followed by surface modification with a capping ligand. For each batch, 8 mg of AuCl₃ was dissolved in 100 ml of DI water while stirring. A 5 mg/ml solution of NaBH₄ was prepared and 2 ml was added in a dropwise manner. After 20 minutes of stirring, 1 ml of a 17 mM

solution of 11-MUA or glutathione was added as capping ligand. The solution was left to incubate overnight at 4 °C. Using 10 kDa molecular weight cut off (MWCO) tubes, and centrifugation at 2500 rcf, the AuNP suspension was concentrated, washed three times with DI water and suspended in DI water to the desired concentration. The final concentration of AuNP was determined using inductively coupled plasma optical emission spectroscopy (ICP-OES) performed on a Spectro Genesis ICP.

Au-PCPP nanoparticle synthesis

For PCPP nanoparticle synthesis, 2 ml of 0.1% PCPP in PBS (PH 7.4) was loaded in a 10 ml syringe. A separate 2 ml solution of 2 mg spermine and 0–0.2 mg PEG-PLL in PBS was prepared and loaded in a second 10 ml syringe. The PEG-PLL amount determines the size of the PCPP nanoparticles formed. The two syringes were attached *via* polytetrafluoroethylene (PTFE) tubing (ID: 0.5 mm, OD: 1 mm, microfluidic ChipShop, Jena, Germany) to a microfluidic chip (Herringbone Mixer, microfluidic ChipShop, Jena, Germany) with two 200 μm $\times$ 300 μm inlets, a 200 μm $\times$ 600 μm staggered herringbone mixer (SHM) channel, and a 200 μm deep $\times$ 600 μm outlet directed to a conical tube (Figure S1). The syringes were loaded on syringe pumps and the solutions mixed at a combined speed of 12 ml/min. The resulting solution was collected in the conical tube and immediately transferred to a 100 ml stirred solution of 8.8% (w/v) CaCl_2 in DI water. After 30 minutes of stirring, the solution was transferred to conical tubes and centrifuged at 600 rcf for 10 minutes. The supernatant was discarded and the pellet was re-suspended in DI water. After repeating this process three times, the PCPP solution was re-suspended in DI water and stored at 4 °C. For Au-PCPP nanoparticles, the 0.1% PCPP solution was mixed with the desired amount of AuNP suspension solution prior to being loaded into the syringe.

Nanoparticle characterization

Transmission electron microscopy (TEM) images were acquired at 80 keV with either a Tecnai T12 microscope (FEI, Hillsboro, OR) equipped with a bottom mounted US1000 2 K $\times$ 2 K CCD camera with a 14 μm pixel size (Gatan Inc, Pleasanton, CA), or a JEM 1010 microscope (JEOL, Tokyo, Japan) with a side entry 1 K $\times$ 1 K video rate camera with a 7.2 μm pixel size (Hamamatsu Photonics, Hamamatsu, Japan). Scanning electron microscopy (SEM) images were acquired with a Quanta 250 (FEI, Hillsboro, OR) at near atmospheric pressure using a gaseous secondary electron detector. Average nanoparticle diameters were measured manually in ImageJ software (NIH, version Java 1.6.0_20 64-bit) for 50–100 nanoparticles from TEM images at various magnifications. UV-Vis spectra were recorded on an Evolution 201 spectrometer (Thermo Scientific, Waltham, MA). A FEI Quanta 600 FEG ESEM equipped with an Octane Silicon Drift detector (EDAX, Inc.) was used for the energy dispersive x-ray spectroscopy (EDS) analysis (using TEAM EDS software). Gold concentrations were determined using ICP-OES.

***In vitro* biocompatibility**

J774.1, HEPG2, Renca and SVEC4-10 cells lines were cultured according to the supplier's instructions. For assessing the biocompatibility of AuNP and PCPP degradation products an MTS assay kit (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega Corporation, Madison, WI) was used. 5 k cells/well were cultured in 96-well plates. After 24

hours, cells were treated with the agent in cell culture media at concentrations of 0, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg/ml. After incubation for 8 hours, cells were washed with DPBS and 120 μ l of a mixture of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and phenazine methosulfate (PMS) (20:1 v/v %) was added into each well. Wells were incubated for ~1 hour at 37 °C, 5% CO₂. The well plate was read at 490 nm using a Synergy Mx microplate reader (BioTek, Winooski, VT). For cell viability with PCPP degradation products, cell culture media was incubated with Au-PCPP at the concentration of 0.5 mg/ml for 1 hour, 1 day or 1 week at 37 °C, the cells were incubated with the aged culture media for 24 hours, and then cell viability assessed using the MTS assay as detailed above.

Since incubation of cells with Au-PCPP could result in the particles sedimenting at the bottom of the cell culture well plate and interfering with the UV-Vis absorbance in the MTS assay, the LIVE/DEAD assay, in conjunction with fluorescence microscopy, was used for assessing cytotoxicity with Au-PCPP. Cells were plated in 35 mm dishes with 20 mm bottom wells at 40 k per well. After 24 hours, cells were treated with Au-PCPP in cell culture media and incubated at 37 °C, 5% CO₂ for 24 hours. After treatment, cells were washed with DBPS twice and incubated for 20 minutes with 400 μ l of LIVE/DEAD cocktail (0.1% stock Ethidium-1 homodimer, 0.025% stock Calcein AM and 0.025% 3.2 mM Hoechst 33342 in DPBS). Four images per well were acquired using three excitation/emission filter pairs: 350/461 nm for Hoechst 33342, 495/515 nm for Calcein and 528/617 nm for Ethidium Homodimer-1 (EthD-1). A custom MATLAB code was used to count the number of cells for each filter pair in each image and the ratio of living cells to total number of cells was calculated as the viability measurement.

Au-PCPP degradation and AuNP release

Au-PCPP (0.2 mg Au) was added to 1 ml of PBS with 10% FBS in micro-centrifuge tubes and incubated at 37 °C for 1, 2, 4, and 7 days. 3 samples per time point were prepared. After the desired incubation time, the tubes were centrifuged at 600 rcf for 10 minutes. The supernatant was collected and the concentration of gold in the supernatant measured using ICP-OES. For TEM and SEM of the degraded Au-PCPP, the pellet from the sample incubated for 7 days was acquired and re-suspended in DI water by washing it using a MWCO tube.

J774A.1 cells were cultured in 6 well plates at ~1 M/well and incubated with Au-PCPP loaded with 11-MUA coated AuNP (0.02 mg/ml cell culture media). After 24 hours, the cells were washed with PBS and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde. Samples were then stained, embedded and cut into 60 nm thick sections and mounted onto TEM grids.

CT phantom imaging

Solutions of Au-PCPP at different concentrations (n=3 per concentration) were suspended in 1% agar gel. 200 μ l of each sample was pipetted into a 250 μ l vial and sealed using Parafilm to prevent water from entering the vials. Vials were secured in a plastic rack and submerged in a plastic container filled with tap water for CT imaging. CT images were acquired using a

clinical SOMATOM Force CT scanner (Siemens Healthcare GmbH, Erlangen, Germany) using a single source abdomen protocol at 80 kV and 177 mA with a reconstructed slice thickness of 0.5 mm, increment of 0.5 mm, and field of view matrix of 512×512 using a smooth kernel (BR40d) with a diameter of 300 mm. The images were analyzed using OsiriX (v.3.7.1 64-bit software). A circular ROI was drawn on the coronal view of each tube and the mean attenuation value for the ROI of three slices per tube was recorded and normalized to the value for PBS. The reported attenuation value for each concentration was the average of three samples of that concentration.

CT *in vivo* imaging

All animal experiments were performed according to protocols approved by University Laboratory Animal Resources in conjunction with the Institutional Animal Care and Use Committee at University of Pennsylvania. *In vivo* CT images were acquired using a MicroCAT II (Siemens Healthcare GmbH, Erlangen, Germany) at 80 kV and 500 μ A in a 512×512 matrix and 100 μ m slice thickness. C57BL/6J mice (n=4) were anesthetized with isoflurane and pre-scanned. 0.75 mg Au-PCPP in 53 μ l was then injected in the thigh, and mice were scanned again 5 minutes post injection. CT images were analyzed using OsiriX. An ROI was placed on the injection site for three CT slices per leg. Mean CT attenuation of the ROI's was normalized to the average attenuation of the pre-scan muscle and the average value per leg was reported.

PA phantom imaging

A PVCP block with small cylindrical cavities (diameter 6 mm, depth 3 mm) was manufactured by casting a heated solution of 60 ml PVCP with 12 g of TiO_2 , added as scattering agent, into a lubricated metal mold.³⁹ The cavities in the block were filled with 100 μ l of Au-PCPP, AuNP or degraded Au-PCPP samples suspended in 1% agar gel at different concentrations (n=3 per concentration). PA images of the vertical cross-section of each cavity was acquired using a Vevo LAZR photoacoustic imaging system (FujiFilm VisualSonics Inc. Toronto, Ontario, Canada) with the laser frequency set at 700 nm. Images were exported as raw images, converted to Tagged Image File Format (TIFF) in MATLAB (v.R2013b) using a code provided by the PA system manufacturer. ImageJ software was used for image processing of the TIFF images. An ROI was placed on the cavity section of the image and the grey scale value of the intensity recorded. The average values for each concentration were normalized to the value for DI water.

PA *in vivo* imaging

C57BL/6J mice (n=4) were anesthetized with isoflurane and 7.5 μ g Au-PCPP was injected in the muscle of the right leg. Three transverse PA images of each leg were then acquired at 20 minutes post-injection and processed as detailed above. An ROI was placed on the injection site for each image and the grey scale intensity of the ROI was measured and averaged for three images per animal. These values were normalized to the intensity values for the non-injected leg.

Results

AuNP synthesis, characterization and biocompatibility

AuNP were synthesized *via* reduction of gold chloride with sodium borohydride. Glutathione and 11-MUA were used as ligands to cap the AuNP for stabilization of the gold cores. Nanoparticles capped with such ligands have been shown to be biocompatible and to be excretable *via* the kidneys.^{25,40–43} The synthesis resulted in cores in the size range of 2–5 nm (Figure S2). The size of the AuNP can be controlled by varying the amount of ligand added before sodium borohydride (Figure S3). For example, 2.2 $\pm$ 0.5 nm AuNP can be synthesized by adding the ligand before sodium borohydride, and 5.1 $\pm$ 1.1 nm AuNP can be synthesized by adding the ligand after the sodium borohydride. To assess the biocompatibility of these nanoparticles, they were incubated with HepG2 (human hepatocytes), SVEC4-10EHR1 (mouse endothelial cells) and Renca (mouse epithelial kidney cells) and their effects on cell viability were assessed. Hepatocyte, endothelial, and kidney cells were examined as these cells should have the greatest exposure to the AuNP upon injection (*i.e.* liver, kidney, and blood vessel linings). Incubation for 8 hours with AuNP did not affect cell viability for these cell lines (Figure S4) at concentrations up to 1.0 mg Au/ml. Furthermore, when cells were incubated with AuNP for 8 hours and evaluated at later time points, no significant change in cell viability was detected in observations made up to a week post incubation (Figure S5). These results, which are in line with previous studies^{40,41} attesting to the inert and non-toxic properties of AuNP designed for biomedical applications, confirm that the AuNP cores are biocompatible.

Au-PCPP synthesis and characterization

We have previously reported the synthesis of polyphosphazene nanospheres by crosslinking the polycarboxylate PCPP with spermine and stabilizing with CaCl_2 ; however, this approach resulted in a high polydispersity of nanoparticle sizes (Figure 2A).⁴⁴ For the work described herein, we developed an improved synthesis method by using a microfluidic device and incorporating a PEG-PLL block co-polymer in order to create more homogenous nanoparticles of tunable size. The microfluidic device uses a staggered herringbone patterned microfluidic chip for nanoparticle synthesis (Figure S1). The staggered herringbone design of the mixing chamber in the microfluidic chip creates a steady chaotic flow through repeated sequences of rotational and extensional local flows which mix the input streams efficiently and homogeneously.⁴⁵ We found that control over the nanoparticle size could be achieved by adjusting the amount of PEG-PLL added. Addition of 0 to 0.1 mg PEG-PLL per mg PCPP in the formulation resulted in PCPP nanoparticles in the size range of 40–500 nm (Figures 2B–C). Inclusion of PEG-PLL in the formulation was found to limit the growth of the PCPP particles, resulting in smaller PCPP nanoparticles with increasing amounts of PEG-PLL.

AuNP can be incorporated into PCPP nanospheres to form Au-PCPP nanoparticles by adding AuNP to the PCPP solution before mixing with spermine. AuNP with both capping ligands were successfully incorporated into PCPP nanospheres, resulting in Au-PCPP particles. As can be seen in Figure 3A, AuNP (dark dots) are incorporated into the PCPP nanoparticles (light gray spheres). Further evidence of AuNP incorporation was given by

EDS measurements (Figure S6). The yield of AuNP inclusion varied between 58 and 71%, as determined by ICP-OES (Figure S7), and was independent of the amount of AuNP added. To assess the loading capacity of Au-PCPP, increasing amounts of glutathione coated AuNP were loaded in PCPP nanospheres (Figure 3A–C). Au-PCPP with a mean size of ~100 nm could carry payloads of glutathione coated AuNP as high as 5 mg AuNP per mg PCPP, without resulting in nanoparticle deformation. Loading AuNP above a certain threshold results in non-spherical or deformed Au-PCPP particles (Figure S8). Similar to PCPP nanoparticles, the Au-PCPP nanoparticles can be tuned in size by controlling the amount of PEG-PLL added to the formulation. Addition of PEG-PLL in the amounts of 6.25 to 25 µg/mg PCPP in a formulation synthesized using 11-MUA coated AuNP resulted in Au-PCPP nanoparticles shown in Figure 3D–F. Loading the PCPP with AuNP increases their size up to 40% as compared to the non-loaded PCPP nanoparticles with the same PEG-PLL amount (Figure S9). This increase in size depends on the AuNP payload and the Au-PCPP size; however, Au-PCPP nanoparticles of a specific target size can still be synthesized by adjusting the amounts of PEG-PLL added to the formulation. The maximum payload of AuNP likely depends on the type of AuNP being loaded into the PCPP nanoparticles. Such experiments will be the topic of future work.

Au-PCPP optical characteristics

AuNP, like other metal nanoparticles, experience the surface plasmon resonance (SPR) effect, which creates a strong optical absorption.⁴⁰ The SPR effect in AuNP depends on their size, morphology, coating ligand, solvent and the interparticle distance.³⁹ Because SPR frequency is dependent on the distance between nanoparticles, aggregating or clustering the nanoparticles can shift their absorbance towards higher wavelengths (a red-shift).⁴⁶ UV-Vis measurements of Au-PCPP revealed red shifts of their absorbance into the near infrared (NIR), compared to the absorption peak of the free AuNP (Figure 4A). Unloaded PCPP nanoparticles at the same polymer concentration as Au-PCPP have minimal absorbance in the 400–900 nm range. Since some of the applications of AuNP in medicine (e.g. photoacoustics, photothermal ablation) depend on absorbance in the near-infrared, this is an appealing feature. We observed a larger red-shift in 11-MUA coated AuNP compared to glutathione coated AuNP when clustered into PCPP to form Au-PCPP particles, therefore we proceeded with Au-PCPP loaded with 11-MUA coated AuNP. Furthermore, this shift in UV-Vis absorption peak is dependent on the size of the Au-PCPP and is linearly correlated with the amount of AuNP loaded into the PCPP nanoparticles (Figure 4B). However, as mentioned above, there is a certain AuNP loading threshold for a given size of PCPP nanoparticles. Therefore, we probed for a formulation that provides absorption in the near infrared, was not overly large, such that it might become entrapped in capillaries (i.e. <400 nm), while retaining structural integrity. For further investigation, we selected the formulation with the 9 µg PEG-PLL/mg PCPP that has a diameter of ~200 nm, and when loaded with 2 mg Au/mg PCPP has an absorption maxima above 650 nm (Figure 4B). A higher 11-MUA coated AuNP loading in this size of Au-PCPP results in deformed nanoparticles (Figure S8). Figures 4C and 4D show the electron microscopy images of this selected formulation. Unless otherwise specified, this formulation was used to assess the *in vitro* and *in vivo* properties of the Au-PCPP nanoparticles that follows.

Au-PCPP biocompatibility

To assess the biocompatibility of the selected Au-PCPP nanoparticles, they were incubated with HepG2, SVEC4-10EHR1 and Renca cell lines. We chose doses that would span the range of what might be expected *in vivo* (for example, at the type of dose used for CT imaging, e.g. 100 mg Au/kg, the blood concentration will be approximately 1 mg/ml immediately after injection and will drop over time). Incubation for 24 hours with Au-PCPP did not affect the viability of these cell lines (Figure 5A). To screen toxicity of the Au-PCPP breakdown products, Au-PCPP nanoparticles were aged in cell culture media at 37 °C for up to a week and the resulting media incubated with cells. Figure 5B shows that Au-PCPP degradation products did not have a significant effect on cell viability. Overall, these results indicate the excellent biocompatibility of this platform.

Au-PCPP biodegradation

The Au-PCPP nanoparticles are very stable outside the body. TEM performed on Au-PCPP stored at 4 °C for six months after synthesis showed no change in the morphology of these nanoparticles (Figure 6A), attesting to their reliable shelf life. Furthermore, UV/vis spectra of a one year old sample showed only minor differences from a freshly synthesized sample (Figure 6B). We investigated the breakdown of Au-PCPP in the presence of biological media. We evaluated the gold core release *via* incubating Au-PCPP in 10% serum in PBS at 37 °C for 7 days and sampling the solution periodically. The solution was centrifuged and the pellet was studied with TEM, while the supernatant was analyzed for free AuNP content. SEM and TEM images of these particles after 7 days of incubation showed the degradation of PCPP polymer and release of AuNP from the nanoparticle structure into the solution (Figure 6C,D). ICP-OES analysis of the supernatant indicated the release of gold, providing evidence for the biodegradability of Au-PCPP. 10% and 15% release of total gold in 7 days was observed for 11-MUA coated and glutathione coated AuNP respectively (Figure 6E). These results demonstrate the degradability of Au-PCPP and subsequent slow release of AuNP. Gold nanoparticles may be released from the PCPP polymer matrix by several mechanisms, i.e. diffusion out of the structure, by solubilization of the polymer through exchange of crosslinking spermine or Ca^{2+} cations with monovalent Na^+ or K^+ or by hydrolysis of the polymer to soluble small molecules.³² We expect that this platform can be engineered to degrade at a desired rate by varying different parameters of the formulation such as the size, loading, and coating of the AuNP and molecular weight of the PCPP. For example, Figure 6F indicates that 50 nm Au-PCPP nanoparticles release gold cores 2.8 times faster than 200 nm Au-PCPP nanoparticles. It is important for the AuNP cores to keep their integrity after being released from the PCPP nanospheres. TEM images show that the AuNP released from Au-PCPP after degradation of PCPP in a mildly acidic buffer (pH 6.0) maintain their integrity (Figure 6G). TEM on macrophages incubated with Au-PCPP for 24 hours revealed that while some particles remained intact (arrowheads), others degraded significantly (asterisks), leaving dispersed gold cores within the endosomes of cells (inset), as can be seen in Figure 6H. Further support for Au-PCPP degradation *in vitro* was given by UV/vis measurements of macrophages, where the absorption maximum was found to be at 560 nm, much less than before the incubation, which would indicate Au-PCPP disassembly. When macrophages were incubated with Au-PCPP for 24 hours, then incubated with fresh media, we observed release of the gold into the media over time, indicating that the Au-

PCPP will be excreted from reticuloendothelial system (RES) cells, should they be phagocytosed (Figure 6I). 66% of the gold that had been taken up was released over 8 days.

CT Imaging

To assess the CT contrast properties of Au-PCPP, phantom samples were prepared at seven different concentrations. A CT scan of these samples in 250 μ l micro-centrifuge tubes submerged in water was acquired using a Siemens Force clinical scanner at 80 kV and 177 mA (Figure 7A). Au-PCPP samples produced strong contrast that was linearly correlated ($R^2=0.96$) with Au concentration as expected (Figure 7B). The attenuation rate of these nanoparticles was 52.3 HU/mg, which is approximately in line with the attenuation rate of 3.6 nm AuNP reported previously.¹² To evaluate the *in vivo* contrast properties of Au-PCPP, a small dose (0.75 mg Au) was injected in the thigh muscle of mice and CT images were acquired before and immediately after injection using a MicroCAT II preclinical scanner at 80 kV (Figure 7C). The amount of time between the two scans was 10 minutes. An ROI was placed on the site of injection and the average value of the HU attenuation for the ROI in three slices per animal was normalized to the pre-scan HU value. The CT contrast enhancement at the injection site was measured to be 4350 ± 1698 HU (n=4) when normalized to the value of the muscle from pre-injection scan (Figure 7C). This small dose of Au-PCPP produced such a strong CT signal due to the small injection volume used and likely scant diffusion from the injection site within the experiment timeframe.

PA Imaging

To assess the PA contrast properties of Au-PCPP, phantoms formed from PVCP were prepared using various concentrations of Au-PCPP or AuNP.³⁹ The PA images of these phantoms were acquired using a laser frequency of 700 nm (Figure 8A,B). Au-PCPP samples, however, produce a significantly higher PA signal due to the red shift of their UV-Vis spectra. This PA intensity increases with concentration up to the concentration of 10 μ g/ml, at which point it reaches a plateau. The AuNP samples did not produce a significant PA signal, likely due to their low absorption of infrared light, as shown in Figure 4A. The low signal produced by these NP prevents any trend in signal from being determined. Similar results to our Au-PCPP data were observed by Orza *et al* who studied the PA characteristics of gold nanoparticles coated with silver iodine.⁴⁷ As the concentration of the contrast agent increases, higher absorption of the light from the particles at the surface of the sample limits light penetration into deeper layers, resulting in the photoacoustic signal leveling off at this higher concentrations. An artifact of this effect is visible as the high intensity of the PA signal at the top layer of both AuNP and Au-PCPP samples (Figure 8B). Measures such as adjustment of gain with the sample depth might yield a linear relationship between contrast observed and concentration over a wider range.

When Au-PCPP samples were degraded in a mildly acidic buffer (pH 6.0) to release the AuNP, their PA contrast potential was significantly reduced (Figure S11). A sample of Au-PCPP loaded with 11-MUA coated AuNP was used to assess the *in vivo* contrast properties of these particles for PA imaging. A low dose (75 μ g Au) was injected in the thigh muscle of mice (n=4). The injection site could easily be identified. The increase in PA contrast in the injected region compared to the non-injected leg was found to be 16.4 ± 4.1 dB (n=4). The

PA images were acquired 20 minutes after the injection of the agent. There is likely little degradation of the agent within this timeframe (see Figure 6), however at significantly longer post-injection timepoints, degradation might affect contrast production. We monitored the mice for up to 3 months post-injection. We did not observe any negative effects on the animals' health such as limping or reduced activity.

Discussion

We have demonstrated that Au-PCPP nanoparticles are biodegradable contrast agents. These nanoparticles, their degradation products and the AuNP cores have no effect on the viability of various cell lines. We chose PCPP as a matrix for this platform because it is biodegradable, biocompatible and can temporarily contain, but subsequently release, hydrophilic nanoparticles. Furthermore, polyphosphazenes have a good track record, since they have been used in many biomedical applications and have been used safely in clinical trials.^{48–51} Modifications of our synthetic approach may allow the use of other polymers that include many carboxylic acid functionalities. For example, alginate may be a promising alternative to PCPP, although obstacles such as lack of synthetic control over its molecular weight and composition and its slow degradation may present challenges. The PCPP-NP are formed via electrostatic and hydrogen bonding interactions between the carboxylic acid groups of PCPP and the amines of spermine. This creates an environment suitable for the inclusion of hydrophilic substances. For example, we have previously reported that PCPP can be loaded with proteins or nanocrystals loaded with phospholipids.⁴⁴ In the current instance, the AuNP used are coated with ligands that possess carboxylic acid functionalities that likely bind to spermine with the PCPP-NP via hydrogen bonding and electrostatic interactions.

Other groups have developed agents for biomedical applications based on aggregates of gold nanoparticles.³⁰ For example, Huang *et al.* reported vesicles composed of AuNP measuring 26 nm in diameter, which are too large for renal clearance. In comparison with previous work, the platform described herein is the first to encapsulate sub-5.5 nm, and thus highly excretable, cores and to demonstrate good synthetic control over the size of the aggregate, which is extremely useful in designing formulations for specific applications. For example, we have shown that the NIR absorption of the nanoparticles can be tuned by controlling the size of the polymer nanoparticles and by the amount of AuNP loading. We demonstrated that Au-PCPP in the size range of 150–200 nm, loaded with AuNP in the amount of 2 mg Au/mg PCPP, have a red shift in the absorption spectra and potential as photoacoustic contrast agents. This size of Au-PCPP was chosen as they are comparable to clinically available nanoparticle contrast agents such as dextran coated iron oxides for magnetic resonant imaging (MRI).⁵² Our experiments, to the best of our knowledge, represent the first to demonstrate tunable syntheses of polyphosphazene based nanoparticles. This is also the first data on biodegradable polyphosphazene nanoparticles, and one of the first reports on the use of polyphosphazenes as delivery vehicles for contrast media.

Because of the facile synthesis procedure, different parameters may be varied for the optimization of the nanoparticles for different applications. As we have previously shown, PCPP nanoparticles can be modified to include other payloads as alternatives or in

combination with AuNP.⁴⁴ Such payloads may include contrast agents for other imaging modalities (such as iron oxide nanoparticles for MRI), nanoparticles with different X-ray attenuations for dual energy CT, or therapeutic agents.

For future work, variation of synthetic parameters will be studied to achieve Au-PCPP with faster biodegradation. These variations consist of using different crosslinkers, PCPP with different molecular weights or alternative ligands for coating AuNP. Following the development of a platform with optimized biodegradation, thorough analysis of pharmacokinetics, *in vivo* toxicological studies (e.g. histology, liver enzymes, oxidative stress, immunogenicity, etc) and assessment of excretion over a range of timepoints will be required before translation of the formulation into the clinic.

Conclusions

Gold nanoparticles are potent contrast agents for various medical imaging modalities. To address the excretion of AuNP larger than 5.5 nm, we proposed a platform where AuNP smaller than 5.5 nm are incorporated in a biodegradable polymer, PCPP, to form Au-PCPP nanoparticles. We have shown Au-PCPP to have a key advantage of larger AuNP, i.e. near infrared absorbance and demonstrated that these larger nanoparticles are biocompatible with different cell lines and degrade *in vitro*. They are potent contrast agents for CT and PA imaging *in vitro* and *in vivo*. Thus the Au-PCPP nanoparticles represent a biocompatible and biodegradable platform with enhanced potential for translation to clinical applications. Although we have not demonstrated the application of these nanoparticles for therapeutic purposes here, this platform may also be suitable for applications such as radiosensitization or photothermal ablation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic depiction of biodegradable gold nanoparticles. Small AuNP are incorporated into a biodegradable polyphosphazene (PCPP) resulting in larger nanoparticles with potent contrast agent imaging capabilities. These larger nanoparticles degrade *in vivo* and release the smaller AuNP for excretion.

Figure 2.

A) TEM image of PCPP nanoparticles synthesized without a microfluidic device (bulk synthesis) showing the polydispersity of their sizes. B) Size variation of PCPP nanoparticles synthesized using the microfluidic device as a function of PEG-PLL amount in the formulation. C) TEM images of PCPP nanoparticles synthesized using a microfluidic device with varying amounts of PEG-PLL added in the formulation (presented as mg PEG-PLL per mg of PCPP).

Figure 3.

TEM images of Au-PCPP synthesized using microfluidics. A–C) 100 nm Au-PCPP loaded with 0.5 mg (A), 2.5 mg (B) and 5 mg (C) of glutathione coated AuNP per mg PCPP. D–F) TEM images of Au-PCPP loaded with 11-MUA coated AuNP synthesized using 25 µg (D), 12.5 µg (E) and 6.25 µg (F) PEG-PLL per mg PCPP in the formulation.

Figure 4.

Selection of a formulation for PA imaging. A) When AuNP are aggregated into Au-PCPP particles, their UV-vis spectra shifts into the NIR region. B) UV-vis measurements of the 11-MUA coated Au-PCPP revealed increasing red shifts into the near infrared by increasing the AuNP loading and size of Au-PCPP. The shift in UV-vis absorption peak is linearly correlated ($R^2 > 0.94$) with the amount of AuNP loaded into the PCPP nanoparticles. C) TEM image and D) SEM image of the selected formulation i.e. 9 μ g PEG-PLL/mg PCPP loaded with 2 mg Au/mg PCPP.

Figure 5.
In vitro biocompatibility of Au-PCPP. A) Viability of cells incubated with varying concentrations of Au-PCPP. B) Viability of cells incubated for 8 hours with cell culture media aged with Au-PCPP for different time spans.

Figure 6.

A) TEM of Au-PCPP 6 months after synthesis. B) UV/vis spectra of freshly made and one year old Au-PCPP samples. C) SEM and D) TEM of Au-PCPP after 7 days incubation with 10% serum (37 °C). E) Degradation of Au-PCPP in 10% serum (37 °C). F) Effect of Au-PCPP size on degradation. G) TEM of glutathione coated AuNP after release from PCPP. H) TEM of macrophage cells incubated with Au-PCPP for 24 hours showing the release of AuNP inside the cells. I) Release of AuNP from macrophages incubated with Au-PCPP.

Figure 7.

CT contrast enhancement with Au-PCPP. A) CT image of Au-PCPP phantoms. B) Linear correlation ($R^2=0.96$) of CT attenuation and Au concentration in Au-PCPP in phantoms. C) Strong CT contrast of Au-PCPP from IM injection in mice.

Figure 8.

PA contrast properties of Au-PCPP loaded with 11-MUA coated AuNP. A) Relationship between PA contrast and Au concentration in Au-PCPP and AuNP. B) PA intensity of Au-PCPP compared to AuNP. C) PA contrast arising from Au-PCPP injected IM in mice. The grayscale images on the left are ultrasound images acquired simultaneously with the PA images. The PA images are the red images on the left.