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Nucleic Acid-directed Self-assembly of Multifunctional Gold Nanoparticle Imaging Agents¹

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Abstract

Gold nanoparticles have attracted much interest as a platform for development of multifunctional imaging and therapeutic agents. Multifunctionalized gold nanoparticles are generally constructed by covalent assembly of a gold core with thiolated ligands. In this study, we have assembled multifunctionalized gold nanoparticles in one step by nucleic acid hybridization of ODN (oligodeoxynucleotide)-derivatized gold nanoparticles with a library of pre-functionalized complementary PNAs (peptide nucleic acids). The PNAs were functionalized by conjugation with DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) for chelating ⁶⁴Cu for PET imaging, PEG (polyethylene glycol) for conferring stealth properties, and Cy5 for fluorescent imaging. The resulting nanoparticles showed good stability both *in vitro* and *in vivo* showing biodistribution behavior in a mouse that would be expected for a PEGylated gold nanoparticle rather than that for the radiolabelled PNA used in its assembly.

Keywords

nucleic acid; self-assembling; gold nanoparticles; imaging; multifunctional; biodistribution; positron emission

Introduction

Nanotechnology offers great potential for the development of highly sensitive diagnostic and targeted therapeutic agents. The particular size range of 10-100 nm gives nanoparticles extraordinary loading capacity for therapeutics and/or contrast agents as well as other functions such as targeting. Multifunctional and multimodal nanoparticles have been developed as magnetic resonance imaging (MRI), positron emission tomography (PET), photoacoustic, and optical imaging agents, as well as photodynamic, photothermal, and chemotherapeutic agents among others.¹⁻⁶ Functionalization of nanoparticles is largely carried out by covalent bond formation using classical bioconjugation methods such as amide bond formation, addition or substitution reactions of thiols, hydrazone formation, and metal-thiol reactions, and more recently *via* various click reactions.⁷⁻¹⁰ These strategies

¹This work is dedicated to Dr. Michael J. Welch, who passed away on May 6th, 2012.

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Supporting Information **Available:** Determination of the molar extinction coefficient for the gold NPs and the average ODN loading of the ODN-AuNP, and the fluorescence melting curves for Cy5-PNA-ODN-AuNP.

generally suffer from difficulty in controlling the extent and reproducibility of the reactions and in characterizing the products, making it difficult to precisely control the composition and properties of the nanoparticles. In addition, the covalent nature of the conjugation reactions can inhibit biodegradation of the nanoparticles. Functionality has also been incorporated through weaker and less specific non-covalent self-assembly mechanisms such as electrostatic interactions, micelle or liposome formation, and host guest interactions.¹¹⁻¹⁴ Nanoparticles produced in this way, however, have variable structure and stability, and are less well defined.

A more promising approach to assembling nanoparticles with programmable composition and properties would be to use the higher specificity and tunable stability afforded by nucleic acid hybridization. Nucleic acid hybridization has been extensively used for constructing and assembling complex 2- and 3-dimensional materials, as well as for detecting nucleic acids by changes in the physical properties of the materials generated.¹⁵⁻¹⁹ It has also recently been used to construct nanoparticles for *in vivo* siRNA delivery²⁰⁻²³ and as reporters on nanoparticles for detecting the presence of complementary nucleic acids in cells (nano-flares).²⁴⁻²⁶ Nucleic acid hybridization has also been previously used in pre-targeting strategies to assemble targeting agents with imaging agents at the *in vivo* target site that are aimed at improving specificity.²⁷⁻³⁰ In most, cases, nucleic acid hybridization has been used to attach only one functional entity to a nanoparticle. In principle, however, nucleic acid hybridization could also be used as a stable, but reversible, non-covalent click reaction to rapidly and combinatorially assemble much more highly functionalized nanoparticles containing any desired combination of targeting, imaging, therapeutic, endosome disrupting, and stealth functionalities (Figure 1).

Gold nanoparticles are an ideal platform for nucleic acid directed self-assembly because they can be readily produced in a variety of sizes and shapes, and easily derivatized with thiolated oligodeoxynucleotides (ODNs).^{15, 31-33} Instead of using a functionalized complementary ODN to hybridize to the ODN on the gold NP, we decided to make use of functionalized PNAs (peptide nucleic acid) because their unnatural polyamide backbone confers a number of advantages over ODNs.^{34, 35} PNAs are highly resistant to degradation by biological systems and also protect the DNA or RNA to which they are bound from degradation. They also hybridize to DNA with higher affinity than does DNA making it possible to use shorter nucleic acid sequences for assembly. Most importantly, they can be conveniently synthesized by solid phase automated peptide synthesis, which allows the easy preparation of PNA-peptide hybrids and the incorporation of a wide variety of amino acid analogues and auxiliary agents. Herein, we report the preparation of functionalized gold nanoparticles through nucleic acid-directed assembly of PNA functionalized with PEG, the PET imaging agent ⁶⁴Cu-DOTA, and fluorescent molecules (Figure 2) with ODN-conjugated gold nanoparticles, that remain intact *in vivo*.

Materials and methods

All solvents and chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise indicated. Fmoc-Lys(mtt)-OH was purchased from EMD Chemicals, Inc. Methoxy PEG succinimidyl carboxymethyl ester, MW 2000 (PEG2K-NHS ester) was purchased from JenKem Technology Co., LTD. Cy5-NHS ester was synthesized by Jillian Smith according to a published procedure.³⁶ The MCF-10A cell lines were purchased from ATCC and cultured under the guidelines provided by ATCC. All cell culture media were purchased from Invitrogen, Inc.

1. Size and zeta potential measurements

Samples for transmission electron microscopy (TEM) measurements were dropped onto PELCO formvar/carbon coated copper grids (Ted Pella, Inc.). After 15 min, the excess colloid was removed by filter paper and 2% uranyl acetate was then dropped on the same grid and incubated for another 2 min. Then the excess solution was again removed by filter paper. The resulting grids were imaged by a FEI Spirit Lab6 TEM. Hydrodynamic diameters and zeta potentials were determined by Malvern Zetasizer Nano using a He-Ne laser at 633 nm. Surface plasmon resonance bands of gold nanoparticles were measured by Varian Cary 100.

2. Synthesis of ODN capped gold nanoparticles ODN-AuNP and ODNnc-AuNP

The ODN capped gold nanoparticles were synthesized according to a reported procedure.¹⁵ Briefly, sodium citrate was added to boiling chloroauric acid solution to yield gold nanoparticles about 11 nm in size.³⁷ TGGTGTGCTTTGTGGATG-O-C₃H₆-SS-C₃H₆-OH and ACCACACGAAACACCTAC-O-C₃H₆-SS-C₃H₆-OH were purchased from IDT and reduced with tris(2-carboxyethyl)phosphine at room temperature for one hour to produce ODN-SH and ODNnc-SH respectively. The ODNs were then isolated by ethanol precipitation and redissolved in water. These freshly prepared thiolated ODN or ODNnc solutions were added to the citrate gold nanoparticles (3 nmol per 1 mL of 7 nM citrate-AuNP). After 20 min, 10% SDS solution was added to bring the mixture to 0.1% SDS. Phosphate buffer (0.1 M, pH=7.4) was added to bring the mixture to 0.01 M phosphate and sodium chloride (2.0 M) was added in 30 min intervals to bring the final mixture to 0.3 M NaCl. The final mixture was gently shaken for 24 h in the dark under room temperature. The gold NP were centrifuged (13500 rcf, 30 min; 3×) and resuspended in PBS.

3. Molar extinction coefficient of the gold NP

Citrate-AuNP or ODN-AuNP (10 μ L) was dissolved in 90 μ L of freshly made aqua regia at RT. The mixture was diluted to 5 mL with 1% nitric acid and the gold concentration was analyzed by Perkin Elmer Elan DRC II ICP-MS using Bi as external reference and standard gold solutions. The concentration of gold nanoparticles was calculated as $[Au]/(4N_A r^3/3)$ where $[Au]$ is the concentration of gold from ICP-MS analysis, ρ is the density of gold (19.3g/cm³), r is the average radius of the AuNP measured by TEM (5.1 nm) and N_A is Avogadro's number. The absorbance of the gold nanoparticles was then measured at 520 nm for citrate-AuNP or 524 nm for ODN-AuNP as a function of concentration, and the molar extinction coefficient obtained from a linear least squares fit of the absorbance vs the particle concentration (Figure S1).

4. ODN loading of the ODN gold NP

ODN-AuNP (0.5 mL of 10 nM) was dissolved in 2 mM KCN solution and 1 μ L of this solution was mixed with 1 μ L of [³²P]-ATP, 2 μ L of T4 polynucleotide kinase buffer A, 1 μ L of T4 polynucleotide solution and 15 μ L of water, incubated at 37 °C for 1 h and then boiled for 10 min. Authentic ODN of known concentration was also labeled following the same procedure. ³²P-labeled ODN (50 nM) and 1 μ L of the radiolabeled ODN from the ODN-AuNP were then separately incubated with known amounts of antisense PNA in Tris buffer (10 mM, pH 7.5) and NaCl (50 mM), boiled for 5 min and slowly cooled down to room temperature. The mixtures were electrophoresed on a 10% native PAGE and the radiolabeled bands were imaged on a Biorad Personal Molecular Imager and quantified by Biorad Quantity One software. A calibration curve was obtained by a linear least squares fit of the observed ratio of the shifted ODN-PNA duplex band and free ODN band to the actual ratio (Figure S2). The calibration curve was then used to determine the concentration of ODN obtained from the dissolved ODN-AuNP solution. The average ODN loading per

ODN-AuNP was then determined from the concentration of the ODN divided by the concentration of the ODN-AuNP determined from the absorbance at 520 nm.

5. General procedure for PNA synthesis

All PNAs and conjugates were synthesized on an Expedite 8900 PNA synthesizer on 2 μmol of Fmoc-PAL-PEG-PS according to the standard automated Fmoc PNA synthesis procedure³⁸ utilizing commercial PNA and peptide monomers (Panagene Inc., Korea). Following the final step of synthesis, the resin was washed with dry CH_2Cl_2 ($2 \times 3 \text{ mL}$), followed by drying under a stream of N_2 . The resin was then shaken in a vial with trifluoroacetic acid (160 μL) and m-cresol (40 μL) at room temperature for 1.5 h twice to release and deprotect the PNA. The solution from each deprotection was separated from the resin, combined and added to ice-cold Et_2O (1.5 mL). The resulting precipitate was collected by centrifugation and purified by reverse-phase HPLC on a Waters XBridge Prep C18 column (130 Å) with buffer A (0.1% TFA in H_2O) and buffer B (0.1% TFA in CH_3CN) on a Beckman System Gold instrument equipped with a UV-vis array detector. The flow rate was 2.5 mL/min and the gradient was 1% buffer B/min starting from 5% buffer B. The fractions were collected and concentrated to dryness in a SpeedVac (Savant) and characterized by UV-vis and MALDI-TOF which was carried out on a PerSpective Voyager mass spectrometer with -cyano-4-hydroxycinnamic acid as the matrix and insulin as the internal reference.

6. Synthesis of Cy5-PNA

PNA CATCCACAAAGCACACCA was synthesized with removal of the amino terminal Fmoc group. Cy5-NHS ester (20 μmol) were dissolved in anhydrous DMF (100 μL) and mixed with DIPEA (2 μmol). The mixture was incubated with the solid support under room temperature for 2 h. Then the solid support was separated, washed with DMF. The Cy5-PNA was cleaved and purified by the general procedure described above. MALDI: exact $[\text{M} + \text{H}]^+$ expected 4413.0, found 4415.2.

7. Synthesis of PNA-DOTA and PNA-Cy5

CATCCACAAAGCACACCA-Lys(Mtt) was synthesized with deprotection and capping of the N terminal amine with 5% acetic anhydride and 6% lutidine in DMF. The Mtt protecting group was removed by washing the solid support with 1 mL of 1% TFA in DCM several times, 5 min each time, until no yellow color was detected. The solid support was then washed with DMF until the eluant was no longer acidic, as measured by wet pH paper. For PNA-Cy5 synthesis, Cy5-NHS ester (20 μmol) and DIPEA (2 μmol) were dissolved in 100 μL DMF and incubated with the solid support at room temperature for 2 h. For PNA-DOTA synthesis, tri-*tert*-butyl 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (20 μmol), EDC (20 μmol) and DIPEA (2 μmol) in 100 μL DMF was incubated with the solid support under room temperature overnight. After both syntheses, the reaction mixtures were removed and the solid supports were washed with 1 mL of DMF four times. PNA-DOTA or PNA-Cy5 was cleaved and purified by HPLC as described above. MALDI of PNA-DOTA: $[\text{M} + \text{Na}]^+$ expected 5365.3, found 5364.8. PNA-Cy5 did not give rise to an identifiable MALDI peak but was characterized by UV, fluorescence spectroscopy, and ability to hybridize with complementary ^{32}P -labeled ODN by native PAGE. UV: A₆₄₂ (Cy5)/A₂₆₀ (PNA), obsvd. 1.13, calcd. 1.37 using estimated extinction coefficients for Cy5 at 642 nm (250,000 $\text{L}\cdot\text{M}^{-1}\cdot\text{cm}^{-1}$) and PNA at 260 nm (182,000 $\text{L}\cdot\text{M}^{-1}\cdot\text{cm}^{-1}$).

8. Synthesis of PNA-PEG₃

CATCCACAAAGCACACCA-Lys(Mtt)-bAla-bAla-Lys(Mtt)-bAla-bAla-Lys(Mtt) was synthesized followed by removal of the amino terminal Fmoc group, the amino terminus

was acetylated with 5% acetic anhydride and 6% lutidine in DMF. The PNA was cleaved from the solid support and purified by HPLC. PEG2K-NHS ester (10 μ mol) was dissolved in 100 μ L DMF and mixed with 1 μ L of DIPEA. Ac-PNA-(Lys-bAla)₂Lys (0.0963 μ mol) was dissolved in 10 μ L DMF and added to the PEG solution, 1 μ L at a time. Following each addition, the pH of the reaction of the mixture was checked with wet pH paper and additional DIPEA was added to maintain the basicity. The reaction mixture was incubated at 55°C for 20 min before the next aliquot was added. After all the PNA was added, the reaction mixture was left under room temperature overnight and then precipitated with ether. The crude product was purified by HPLC. PNA-PEG3 did not give rise to identifiable MALDI and was analyzed by its ability to hybridize with ³²P labeled ODN by native PAGE.

9. Fluorescence quenching studies

Various amounts of 36.6 μ M Cy5-PNA were added to 60 μ L of 5.23 nM ODN-AuNP or ODNnc-AuNP or citrate-AuNP in 1X PBS. The mixtures were incubated at room temperature for 5 min and then the fluorescence was measured at 663 nm with excitation at 633 nm. The fluorescence of Cy5-PNA in 1 $\times$ PBS was measured at each concentration as control.

10. Fluorescence melting temperature study of Cy5-PNA-ODN-AuNP

Temperature-dependent fluorescence intensity measurements of Cy5-PNA-ODN-AuNP were made with excitation at 633 nm and emission detection at 663 nm. The Cy5-PNA-ODN-AuNP complex (0.27 μ M Cy5-PNA, 4.7 nM ODN-AuNP in 1X PBS buffer) was heated from 25° to 80 °C at 1 °C per min and held at 80 °C for 5 min and then cooled down to 25 °C (Figure S3A). After 5 min, the heating-cooling cycle was repeated. The temperature dependent fluorescence of Cy5-PNA alone was also acquired (Figure S3B). The raw data was then converted to fraction single strand by using the average observed fluorescence for Cy5-PNA-ODN-AuNP as a function of temperature (F_{obsvd}) and using a linear fit of the fluorescence curve prior to melting for the temperature dependence of the duplex form (F_{duplex}), and using the fluorescence curve of the Cy5-PNA for the temperature dependence of the single strand form (F_{single}). The fraction single strand at a given temperature was then calculated as $(F_{\text{obsvd}} - F_{\text{duplex}})/(F_{\text{single}} - F_{\text{duplex}})$. Thermodynamic parameters were derived from Van't Hoff analysis as shown in Fig. S4.

11. Absorbance melting temperature study of Cy5-PNA-ODN

The absorbance at 260 nm of the complex from Cy5-PNA (26.4 μ M) and ODN (40 μ M) in 1 $\times$ PBS buffer was monitored from 25° to 85 °C at 1 °C per min, and after holding at 85 °C for 5 min, from 85° to 25 °C. After 5 min, the heating-cooling cycle was repeated. The data was then converted to fraction single strand by using the average observed absorbance for Cy5-PNA-ODN as a function of temperature (A_{obsvd}) and linear fits to the initial and final parts of the melting curve for the temperature dependence of the duplex (A_{duplex}) and single strand (A_{single}) forms respectively. The fraction single strand at a given temperature was then calculated as $(A_{\text{obsvd}} - A_{\text{duplex}})/(A_{\text{single}} - A_{\text{duplex}})$.

12. Cellular uptake of ODN-AuNP

MCF10A cells were seeded in 35 mm glass bottom dish and allowed to grow to 70% confluence. PNA-Cy5 (1 μ L of 1.18 μ M) and 15 μ L of 10 nM ODN-AuNP was incubated at 50 °C for 10 min and after cooling to RT, was added to 135 μ L of fresh complete media containing 0.5 μ g/mL of FAM-dextran (MW 25,000, Invitrogen). The cell media was then replaced with this mixture and incubated at 37 °C with 5% CO₂ for 6 h. After 5 h, 2 μ L of 10 μ g/mL Hoechst 3342 was added to the mixture. 1 h later, the mixture was removed and

the cells were washed with 1× PBS buffer 5 times and then incubated with 2 mL of fresh media. The cellular uptake was visualized by Nikon A1 confocal microscope.

13. Reduction of nonspecific cell binding by PEGylation

MCF10A cells were seeded in a 12 well plate and grown to 90% confluence. PNA-PEG₃ was mixed with ODN-AuNP in various ratios. The mixtures were incubated at room temperature for 30 min and added to the cells with complete cell media. The final concentration of AuNP was 0.9 nM. After 6 h of incubation at 37°C, cell media was removed and the cells were washed with PBS twice. The cells were trypsinized, counted with hemacytometer and centrifuged. Each cell pellet was digested with 100 µL nitric acid and hydrogen peroxide mixture (9:1, v/v) overnight followed by addition of 200 µL aqua regia. The resulting solution was then diluted to 7 mL with 1% nitric acid and analyzed by Perkin Elmer Elan DRC II ICP-MS using Bi as external reference.

14. Radiolabeling of the PNA

Copper-64 ($t_{1/2}=12.7\text{h}$, $^{+}=17\%$, $^{-}=40\%$) was produced on the Washington University Medical School CS-15 cyclotron by the $^{64}\text{Ni}(p,n)^{64}\text{Cu}$ nuclear reaction at a specific activity of 1.85-7.40 GBq/µg at the end of bombardment.³⁹ PNA-DOTA (320 pmol) was incubated with ^{64}Cu (185 MBq) in 100 µL of 0.1 M ammonium acetate buffer (pH 5.5) at 65°C for 1 h, with a yield of $52.5 \pm 2.4\%$. The PNA-DOTA- ^{64}Cu was purified by solid-phase extraction after ethylenediaminetetraacetic acid (EDTA, 10 mM in 50 mM pH 7.4 phosphate buffer) and the specific activity was 301 ± 14 MBq/nmol.⁴⁰ The PNA-DOTA used for annealing with ODN and ODN-AuNP was radiolabeled and purified following the same procedure with the specific activity of 285 ± 11 MBq/nmol. Then 37 MBq of PNA-DOTA- ^{64}Cu was mixed with ODN. Another 37 MBq (9.1 nmol) of PNA-DOTA- ^{64}Cu was mixed with ODN-AuNP (0.52 nmol) followed by addition of PNA-(PEG2K)₃ (27.3 nmol). The mixture was purified with a Zeba spin desalting column (Pierce).

15. Bio-Distribution studies

All animal studies were performed in compliance with guidelines set forth by the NIH Office of Laboratory Animal Welfare and approved by the Washington University Animal Studies Committee. Biodistribution studies were performed in male C57BL/6 mice weighing 20-25 g ($n=4/\text{group}$) and about 370 kBq of PNA-DOTA- ^{64}Cu , PNA-DOTA- ^{64}Cu -ODN and PNA-DOTA- ^{64}Cu -PEG₃-ODN-AuNP in 100 µL saline (APP Pharmaceuticals) was injected *via* the tail vein. The mice were anesthetized with inhaled isoflurane and were re-anesthetized before being euthanized by cervical dislocation at each time point (1 h, 4 h, and 24 h) post injection (p.i.). Organs of interest were collected, weighed and counted in a gamma counter (Beckman 8000). Standards were prepared and measured along with the samples to calculate the percentage of the injected dose per gram of tissue (%ID/gm).⁴¹

Results and discussion

1. ODN-Gold Nanoparticle Synthesis

Citrate gold nanoparticles, (citrate-AuNP) were synthesized by standard methods³⁷ and were 10.2 ± 0.8 nm in diameter as measured by TEM (Figure 3a). Dynamic light scattering gave a very similar hydrodynamic radius of 10.3 ± 2.6 nm (Figure 4a). The particles had an expected highly negative zeta potential of -22.5 ± 2.8 mV. Conjugation of the gold nanoparticles to a 3 -thiolated ODN, ODN-SH (Figure 2), by standard methods¹⁵, gave ODN-AuNP. TEM of a negatively stained sample showed particles with a diameter of about 16.1 ± 0.9 nm consisting of a dark core and a bright outer circle corresponding to the ODN layer (Figure 3b). Dynamic light scattering gave a similar hydrodynamic diameter of $16.2 \pm$

4.1 nm (Figure 4b). The thickness of the ODN layer was about 2.5-2.8 nm as deduced from the difference in size of the ODN-AuNP and the citrate-AuNP from TEM and DLS experiments. The thickness of the ODN layer is less than half the length of a helical 18-mer ODN (6.3 nm) indicating that the ODN is not fully extended on the gold surface and may be partially contacting the surface. The zeta potential of the ODN-AuNP decreased to -31.7 ± 0.1 mV due to the large number of negatively charged phosphate groups contributed by the ODN.

2. Sequence specific self-assembly with PNA

Gold has been known as an effective fluorescent quencher and the fluorescent quenching efficiency decreases drastically as the distance between the fluorophore and the gold surface increases.⁴² We utilized this unique property to demonstrate the self-assembly of the ODN-AuNP with PNA. We synthesized a 15-mer PNA that was labeled with Cy5 at its amino end, Cy5-PNA (Figure 2), which is complementary (antisense) to the ODN on the AuNP. Upon PNA-ODN hybridization, the fluorophore is positioned three nucleotides away from the gold surface, or about one nm if the PNA-ODN duplex were perpendicular to the surface.⁴³ This distance is close enough to cause significant fluorescent quenching.⁴² As shown in Figure 5, the fluorescence of the Cy5 was almost completely quenched when the Cy5-PNA was mixed with ODN-AuNP and remained quenched until the concentration of the Cy5-PNA-mer was about 90 times the concentration of the gold nanoparticle. Beyond this point, the fluorescence started to increase linearly indicating that all the complementary binding sites on the gold NP had been saturated. The gold NP without the ODN shell, however, also quenched the fluorescence of the PNA. To demonstrate that the quenching was due to sequence specific binding of the PNA to the ODN shell, and not simply to the gold surface, a gold NP bearing the non-complementary ODN, ODNnc (Figure 2), was also synthesized. When titrated with Cy5-PNA very little quenching was observed (Figure 5).

To determine the stability of the PNA-ODN hybrid on the gold NP, temperature dependent fluorescence measurements were made between room temperature and 85°C. Following an initial heating to 85°C to degas the sample, the fluorescent heating and cooling curves for ODN-AuNP-Cy5-PNA were quite reproducible and indicated that the complexes had a melting temperature of 70 °C (Figure 6). This can be compared to a melting temperature of 71.5 °C for ODN-Cy5-PNA determined by temperature dependent absorbance measurements. Thermodynamic parameters for the stability of the two duplexes was obtained from Van't Hoff analysis of the melting temperature curves for a two state all or none melting model for the temperature range of 52-72°C (the most linear increase in G) (Fig. S4). For the free ODN and AuNP bound ODN, H° was -53.2 ± 1.0 and -38.6 ± 1.2 kcal/mole, and S was 133 ± 3 and 82.3 ± 6 cal/deg-mol, respectively. From these values, the K_d 's for PNA binding to the free and AuNP bound ODN at 37°C were extrapolated as 4.2 and 0.7 nM respectively. The K_d for the AuNP bound ODN was about 6 times lower, likely due to the polydentate effect.

3. PEGylation of the ODN-Gold NP

For imaging specific targets *in vivo* it is important that the NPs are not recognized by the MPS (mononuclear phagocyte) system, often referred to in the literature as the RES (reticulo-endothelial) system, or taken up by non-target cells.⁴⁴ It has been found that PEGylation can greatly reduce binding of NP to proteins and confer stealth character.⁴⁵ To study the interaction of the PNA-ODN-AuNP with cells and the ability of PEGylation to reduce non-specific binding and uptake, we synthesized a fluorescently labeled PNA for tracking the NP and a PEGylated PNA for conferring stealth character. To minimize quenching of the fluorescence, Cy5 was conjugated to the carboxy terminus of PNA to give PNA-Cy5 (Figure 2) *via* a carboxy-terminal lysine group so that the fluorophore would be

directed away from the surface of the gold NP. To create a PEGylated PNA with sufficient PEG to cover the surface, the PNA was derivatized with three PEG2K molecules *via* acylation of three lysines at the carboxy terminus of PNA and spaced apart by two beta-alanines to give PNA-PEG₃ (Figure 2). The ODN-AuNP was hybridized with a stoichiometric amount of PNA with differing ratios of PNA-PEG₃:PNA-Cy5. TEM analysis under negative staining of 75/25 ratio of PNA-DOTA/PNA-PEG₃-ODN-AuNP showed a dark center of about 12 nm, representing the gold core, surrounded by a narrower bright band than was seen with the ODN-AuNP, and a large and diffuse dark area of average diameter 41.7 ± 1.1 nm that must correspond to the ODN-PNA duplex linked to the PEG2K layer (Figure 3C).

Dynamic light scattering gave a hydrodynamic radius of 30.2 ± 9.1 nm which when subtracted from the original diameter of 10.1 nm for the citrate-AuNP gives an increased diameter of 17.1, or an increased radius of 8.5 nm (Figure 4). The increased radius corresponds very well to 6 nm for the PNA-ODN helix ($18 \text{ bp} \times 0.33 \text{ nm/bp}$)⁴³ plus 3.8 nm corresponding to the Flory radius of PEG2K.⁴⁶ Simple calculations suggest that there is ample surface area to accommodate the PNA·ODN duplex and the three PEG2K chains. A PNA·ODN duplex has a diameter of 2.2 nm corresponding to a cross-sectional area of 0.95 nm² which when multiplied by 100 ODNs/AuNP gives an area of 95 nm² which is 3 times less than the calculated surface area of 314 nm² for a 10 nm diameter gold particle. If fully extended, the PNA-ODN duplex would extend the radius of the gold NP by 6 nm, resulting in an outer surface area of 1520 nm², or 152 nm² per PNA·ODN duplex. Given that the Flory radius of PEG2K is 3.8 nm and that the center of radius for each PEG2K would be an additional 1 nm further out, then the available surface area is calculated to be 3157 nm². Since three PEG2K accommodate a cross-sectional surface area of 114 nm², the available surface would become saturated upon addition of about 28 PNA-PEG₃ units. Above this amount, one would expect the PEG chains to adopt a more extended, brush-like structure. In accord with this analysis, the zeta potential of the particle increased to -4.1 ± 0.4 mV as the PEG layer significantly shields the charges.⁴⁷

4. Cellular uptake of the PEGylated ODN-Gold NP

The interaction of the AuNPs with a normal human breast cell line (MCF10A) was examined by confocal microscopy and quantified by inductively coupled plasma mass spectrometry (ICP-MS). As expected from previous studies with ODN-AuNP,⁴⁸ Cy5-PNA·ODN-AuNP was internalized by MCF-10A cells efficiently in complete growth media without any cell permeation enhancing ligand (Figure 7A). The particle (red) colocalized with FAM-dextran (green) which is considered to enter the cells through an endosomal pathway. The uptake presumably occurs through specific uptake of serum proteins bound to the gold nanoparticle. PEG is known to prevent protein binding to nanoparticles, and upon complexation with PNA-PEG₃, the uptake decreased with increasing density of the PNA-PEG₃ on the gold NP (Figure 7B). MCF-10A uptake was reduced by 60% at 60% PEGylation and further reduced by 80% with almost complete PEGylation. The required percentage of PNA-PEG₃ could possibly be further driven down by utilizing PEG of higher molecular weight or higher density per PNA.

5. Biodistribution Studies of the PEGylated Gold NP

To determine whether or not the non-covalently assembled PNA-PEG₃-ODN-AuNP would remain intact *in vivo*, we carried out biodistribution studies with a nanoparticle that was radiolabeled with the PET radionuclide ⁶⁴Cu.^{49, 50} To radiolabel the particle, we synthesized a PNA functionalized with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) which has a high affinity for the radionuclide ⁶⁴Cu. The DOTA was coupled to carboxy-terminal lysine on the PNA *via* tri-*t*-butyl-DOTA ester and 1-ethyl-3-(3-

dimethylaminopropyl)carbodiimide (EDC) to give PNA-DOTA (Figure 2) following deprotection. PNA-DOTA was first radiolabeled with ^{64}Cu , purified, and then hybridized with a complementary ODN and the ODN-AuNP.

The biodistribution of the ^{64}Cu -PNA-DOTA associated with the ODN-AuNP (Figure 8C) was strikingly different than for ^{64}Cu -PNA-DOTA alone (Figure 8A), or when complexed to the ODN (Figure 8B) at 1 h, 4 h and 24 h post injection (p.i.) Both ^{64}Cu -PNA-DOTA and ^{64}Cu -PNA-DOTA·ODN were cleared very quickly through the renal system, with some minor accumulation in the liver, lung and gastrointestinal tract (stomach and intestine), and negligible uptake in other organs (Figures 8 A & B). At 1 h p.i., the blood retention of ^{64}Cu -PNA-DOTA and ^{64}Cu -PNA-DOTA·ODN were both less than 0.5 % ID/g while the kidney retention was about 5%. The liver uptake was constant over 24 h with less than 2% ID/g. While ^{64}Cu -PNA-DOTA/PNA-PEG₃-ODN-AuNP also showed rapid clearance from systemic circulation (0.31 ± 0.09 % ID/g at 1 h p.i.), it showed much higher retention in liver than free ^{64}Cu -PNA-DOTA or its ODN hybrid (Figure 8C), typical of covalently linked gold nanoparticles of similar size and composition. For example, the biodistribution at 24 h (21% liver, 11% spleen, 4% kidney) was very similar to that reported in mice at 48 h for a 20 nm gold nanoparticle coated with thiotic acid-anchored PEG5K with a hydrodynamic radius of 45 nm (30% liver, 15% spleen and 5% kidney).⁵¹ While liver uptake was dominant, it gradually decreased over 24 h from $32 \pm 3\%$ ID/g at 1 h, to $28 \pm 7\%$ ID/g at 4 h, and $21 \pm 2\%$ ID/g at 24 h p.i.. The spleen accumulation, however, was stable with about 10% ID/g through the study.

The sharp contrast between the biodistribution profile of ^{64}Cu -DOTA/PNA-PEG₃-ODN-AuNP and ^{64}Cu -PNA-DOTA by itself or when hybridized to the ODN at different time points demonstrates that the nanoparticle remained largely intact during the initial biodistribution. Since it has been established that gold nanoparticles themselves do not clear from the liver for many weeks,⁵² the loss of about 50% of the initial dose that accumulated in the liver at 4 h after 24 h may be due to cleavage of the ODN-Au linkage or dissociation of the PNA·ODN duplex. These two mechanisms could be distinguished in the future by using longer, more stable PNA·ODN duplexes. The pharmacokinetics of ^{64}Cu -DOTA/PNA-PEG₃-ODN-AuNP would be less than optimal for potential *in vivo* therapeutic applications due to the rapid depletion of the nanoparticle from the blood and high accumulation in the liver. The rapid clearance may be useful, however, for some diagnostic applications in which a low background would be desirable. Further surface modification of the nanoparticle to resist protein binding and non-specific uptake by the liver will likely be needed before it can be used for efficient cell specific targeting, and is the focus of our further work.

Conclusion

We have shown that DNA-directed nucleic acid hybridization with PNA can be used for the rapid combinatorial self-assembly of multifunctional gold nanoparticles that largely maintain their integrity *in vivo*. The strategy is not limited to gold nanoparticles, but to any nanoparticle bound to oligodeoxynucleotides or their analogs. Nucleic acid hybridization also provides a simple and straightforward method for radiolabeling of the nanoparticle with a PET imaging agent using a purified ^{64}Cu -labeled PNA. Because the stability of the PNA·DNA hybrids used to assemble the nanoparticle is tunable by sequence and length, the stability of the nanoparticles is also expected to be tunable. These initial results demonstrate that nucleic acid-directed self-assembly could be used for the combinatorial synthesis and optimization of nanoparticle-based imaging and therapeutic agents through what amounts to non-covalent click chemistry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Self-assembly of functionalized gold nanoparticles. Gold nanoparticles conjugated to thiolated ODNs are hybridized with any combination of functionalized PNAs to rapidly assemble a combinatorial library of functionalized gold NPs.

Figure 2. Functionalized ODNs and PNAs used in this study. All ODNs are written in a 5' → 3' direction and all PNAs in an amino → carboxy direction.

Figure 3.

TEM pictures of gold NPs at various stages of construction and assembly. TEM of (a) citrate-AuNP, (b) ODN-AuNP, and (c) PNA-PEG₃/ PNA-DOTA-ODN-AuNP with a PNA-PEG/PNA-DOTA ratio of 3:1. All three samples were stained with uranyl acetate. Panel (c) was assembled from four representative pictures. The scale bars represent 20 nm.

Figure 4. Dynamic light scattering analysis of gold NP at various stages of construction.

Figure 5.

Fluorescence titration of ODN-AuNPs with Cy5-PNAs. A) Fluorescence of increasing concentration of Cy5-PNA in the presence (○) or absence (●) of 5 nM ODN-AuNP. B) Fluorescence of increasing concentration of Cy5-PNA in (○) buffer alone, or the presence of: (◆) 10 nM ODN-AuNP; (▲) 10 nM ODNnc-AuNP; (△) 10 nM citrate-AuNP.

Figure 6.

Melting curves for Cy5-PNA·ODN-AuNP and Cy5-PNA·ODN. The fraction single strand form for Cy5-PNA·ODN-AuNP (●) from analysis of temperature dependent Cy5 fluorescence measurements at 0.270 μM PNA and 0.423 μM ODN bound to AuNP, and for Cy5-PNA·ODN (○) from analysis of temperature dependent absorbance measurements at 26.4 μM PNA and 40 μM ODN. Data from two heating/cooling cycles were used to generate each melting temperature curve. Determination of the thermodynamic parameters for duplex formation can be found in Fig. S4.

Figure 7. Effect of the degree of PEGylation on uptake of PNA-Cy5/PNA-PEG₃-ODN-AuNP by MCF-10A cells. A) fluorescence imaging of MCF10A cells incubated with PNA-Cy5-ODN-AuNP (red) and FAM-dextran (MW 25,000) (green). Cell nuclei were stained with Hoechst 3342 (blue) B) quantification of PNA-Cy5/PNA-PEG₃-ODN-AuNP uptake by cells by ICP-MS.

Figure 8. Biodistribution studies of AuNP and components. A) PNA-DOTA- ^{64}Cu , B) PNA-DOTA- ^{64}Cu -ODN and C) PNA-DOTA/PEG₃- ^{64}Cu -ODN-AuNP for 1h (a), 4h (b) and 24h (c) p.i.