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Labeling of Adenovirus Particles with PARACEST Agents

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Abstract

Recombinant adenovirus type 5 particles (AdCMVLuc) were labeled with two different bifunctional ligands capable of forming stable complexes with paramagnetic lanthanide ions. The number of covalently attached ligands varied between 630 and 1960 per adenovirus particle depending upon the chemical reactivity of the bifunctional ligand (NHS ester versus isothiocyanide), the amount of excess ligand added, and the reaction time. The bioactivity of each labeled adenovirus derivative, as measured by the ability of the virus to infect cells and express luciferase, was shown to be highly dependent upon the number of covalently attached ligands. This indicates that certain amino groups, likely on the surface of the adenovirus fiber protein where cell binding is known to occur, are critical for viral attachment and infection. Addition of $^{177}\text{Lu}^{3+}$ to chemically modified versus control viruses demonstrated a significant amount of nonspecific binding of $^{177}\text{Lu}^{3+}$ to the virus particles that could not be sequestered by addition of excess DTPA. Thus, it became necessary to implement a prelabeling strategy for conjugation of preformed lanthanide ligand chelates to adenovirus particles. Using preformed Tm^{3+} -L2, a large number of chelates having chemical exchange saturation transfer (CEST) properties were attached to the surface residues of AdCMVLuc without nonspecific binding of metal ions elsewhere on the virus particle. The potential of such conjugates to act as PARACEST imaging agents was tested using an on-resonance WALTZ sequence for CEST activation. A 12% decrease in bulk water signal intensity was observed relative to controls. This demonstrates that viral particles labeled with PARACEST-type imaging agents can potentially serve as targeted agents for molecular imaging.

INTRODUCTION

Magnetic resonance imaging (MRI), one of the most widely used noninvasive imaging modalities, provides contrast in images of soft tissues that depend upon the proton density of tissues and inherent differences tissue water relaxation rates. In the early 1980s, it was discovered that paramagnetic metal chelates could accelerate proton spin–lattice relaxation rates and thereby enhance image contrast. More recently, another method for altering MR image contrast was reported that relies on selective presaturation of chemically exchanging protons involving either endogenous molecules or exogenous probes which then exchange with bulk water protons to reduce its intensity. This new class of contrast media is referred to as

CEST agents (1). There are numerous types of exchangeable protons in tissue, but the most common are -OH and -NH groups. Unfortunately, the chemical shift difference between these types of exchanging protons and water protons is small, and consequently selective saturation can be problematical. However, the potential advantages of CEST agents over conventional T_1 agents, including the ability to switch contrast “on” and “off” and the sensitivity of CEST to environmental effects such as pH and temperature, provide incentives for continued development of such agents. The concentration of any single chemically exchanging proton or water molecule that is required to produce significant CEST contrast is quite high, but this limitation can partially be compensated for by optimizing the chemical exchange rate (k_{ex}) between the agent and bulk water and by maximizing the number of exchangeable sites available on each agent. A successful example of the latter was reported recently (2) where glycogen in mouse liver was imaged by presaturation of the exchanging glycogen -OH protons (glycoCEST) that lie ~1 ppm downfield of tissue water.

Paramagnetic metal complexes having a single, slowly exchanging bound water molecule can function by a CEST mechanism as well (3). As the chemical shift of lanthanide-bound water molecules can be shifted as much as 700 ppm away from the bulk water resonance, this offers the possibility of selectively saturating the Ln^{3+} -bound water proton signal with less chance of inadvertently saturating bulk water protons. This large chemical shift difference between the Ln^{3+} -bound and bulk water signals makes this class of CEST contrast agents unique. For this reason, the term “PARACEST” is now used to differentiate such paramagnetic complexes from diamagnetic CEST compounds (4). Although paramagnetic systems have this attractive advantage, they do suffer from the same lack of sensitivity as diamagnetic CEST molecules on a proton concentration basis. One obvious method that could be used to increase the sensitivity of a CEST agent is to increase the number of exchangeable sites per molecule (5). High molecular weight platforms such as proteins, liposomes, linear and branched polymers, and spherical polymers based on core-shell morphology offer the opportunity to accumulate higher numbers of exchangeable sites per particle and thereby increase their “effective” concentration.

There is interest in using viral particles as biodelivery platforms due to their structural and architectural diversity and biological properties. The architecture of the viral particles consists of interior and exterior regions that can be chemically or genetically modified. The interior compartment of the viral particle can provide a naturally constrained cage for encapsulation of inorganic or organic materials (6), while chemical modifications of exterior surface capsid proteins have included specific nucleation of inorganic materials on the surface of the particles, and derivatization with PEG (polyethylene glycol) (7), fluorescent labels (8,9), peptides (10), or MRI contrast agents (11). Studies by Allen et al. (12) have shown that the naturally occurring, endogenous metal binding sites of cowpea chlorotic mottle virus bind Gd^{3+} ions with a dissociation constant of $K_d = 31 \mu M$ yielding a nanoparticle template for MRI applications. Anderson and co-workers (11) have extended this strategy by labeling the MS2 virus capsid with over 500 $GdDTPA$ moieties ($DTPA$ = diethylenetriaminepentaacetic acid) but did not test the viral bioactivity of the resulting modified nanoparticles.

Human adenovirus is a nonenveloped, double-stranded DNA-containing particle approximately 130 nm in diameter with icosahedral symmetry (13). Although over 50 types and 6 subgroups of human adenovirus have been identified, adenovirus type 2 and 5 of subgroup C are most commonly used as vectors in gene transfer. The adenovirus binds to cell surface receptors with high affinity via a COOH-terminal globular knob domain of the fiber protein. The recombinant knob domain binds about 4700 sites per HeLa cell with an affinity of $3 \times 10^9 M^{-1}$ and is capable of blocking adenovirus infection of human cells (14). Each capsid protein of the adenovirus particle contains multiple exposed functional groups that can be chemically modified. Adenovirus 5 knob protein chemically modified with succinidyl 6-

hydrozinonicotinate (HYNIC) and subsequently radiolabeled with ^{99m}Tc shows high-affinity binding to cells ($K_d = 1.4 \text{ nM}$) (15). Replacement of the E1 region of the viral genome with a foreign gene generates a recombinant vector capable of efficiently infecting a wide variety of cells *in vitro* and tissues *in vivo*. These properties have prompted our interest in the use of adenoviral particles as drug delivery platforms.

The goal of this study was to compare the chemical reactivity of two bifunctional chelating agents **L1** and **L2** (Scheme 1) with replication-defective adenovirus particles and to optimize the labeling of viral particles with PARACEST agents derived from thulium (Tm^{3+}) and **L2** under conditions where viral activity is not totally disrupted. The AdCMVLuc virus (20) containing the luciferase reporter gene was chosen for this study so we could follow the infectious activity of the virus using a standard luciferase chemiluminescent assay after chemical modification with either reactive metal-free bifunctional ligands or metal ion preloaded chelates with favorable exchange properties for CEST imaging. Our goal was to create PARACEST-labeled viral particles that retain their infectious biological activity for potential use as tissue targeting agents for combined optical (luciferase) and MR (PARACEST) molecular imaging.

EXPERIMENTAL PROCEDURES

Materials and Methods

All starting reagents and solvents were obtained from commercial sources and used as received unless otherwise indicated. ^1H and ^{13}C NMR spectra were acquired using a JEOL Eclipse 270 operating at 270 and 67.5 MHz, respectively. Chemical shifts are reported in parts per million (δ) relative to TMS at 0 ppm. Infrared spectra were obtained on a Nicolet Avatar 360 FT-IR spectrometer either as KBr pellets or as thin films. UV-vis absorption spectra were recorded using a Pharmacia LKB-Ultrospec III single beam spectrometer. Hydrogenation was performed using a Parr hydrogenation apparatus. Electrospray ionization mass spectroscopy (ESI MS) was performed by HT Laboratories, San Diego, California. Fast atom bombardment mass spectroscopy (FAB MS) was performed by the Mass Spectrometry Facility at the University of Alabama at Tuscaloosa. Elemental analyses and inductively coupled plasma mass spectrometry (ICP MS) measurements were completed by Galbraith Laboratories, Knoxville, Tennessee. Luciferase (relative light units) was quantified using a SIRUS Luminometer V3.1. ^{177}Lu (0.05 N HCl) was obtained from the Research Reactor Center, University of Missouri at Columbia, Missouri. All buffers and reagent solutions for radiolabeling were prepared in Millipore deionized (DI) water and treated with Chelex 100 resin (100–200 mesh, Na^+ form, Bio-Rad Laboratories) for at least 4 h, followed by filtration through 0.22 μm CA membrane (Corning Inc.). Metal-free plastic ware used for all labeling experiments was soaked in 15% HNO_3 for 12 h and rinsed with Millipore DI water prior to use. Quantification of radioactivity was determined using a Rita Star Radioisotope TLC analyzer (Straubenhardt, Germany). Virus concentrations are expressed in particles/mL as measured by absorbance at 260 nm. CEST spectra and T_2 measurements were acquired using Varian INOVA-500 spectrometer at 500 MHz. Prior to data acquisition, samples were allowed to warm to room temperature and equilibrated in the probe at 25 $^\circ\text{C}$ for at least 10 min.

Synthetic Methods

1,4,7-tris(*tert*-butoxycarbonyl)-1,4,7,10-tetraazacyclo dodecane (16), 2-bromo-*N*-methylacetamide, 2-*p* isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(methylcarbonylamide) (17), and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(methylcarbonylamide) (referred to as DTMA) (18) were prepared as described previously.

1,4,7-Tris(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecane-10-benzyl acetate (4)

K₂CO₃ (1.54 g, 11.1 mmol) was added to an acetonitrile (20 mL) solution of tris-Boc-protected cyclen **3** (4.3 g, 9.09 mmol). The suspension was heated for 10 min at 60 °C, followed by addition of benzyl bromoacetate (1.76 mL, 11.2 mmol). The reaction mixture was heated for 12 h at 60–70 °C, then filtered, and the solvents removed under reduced pressure. The residue was purified by column chromatography over silica gel eluting with 10% methanol in chloroform to afford the title compound as a colorless solid (5.2 g, 92% yield). *R*_f = 0.5 (SiO₂, MeOH/CHCl₃, 1:9). ¹H NMR (270 MHz, CDCl₃): δ = 1.42 (18H, s, C(CH₃)₃), 1.44 (9H, s, C(CH₃)₃), 2.9–3.2 (16H, s br, ring CH₂), 3.5 (2H, s, CH₂CO₂), 5.1 (2H, s, OCH₂Ph), 7.3 (5H, m, Ph). ¹³C NMR (67.5 MHz, CDCl₃) δ = 28.4 C(CH₃)₃, 28.7 C(CH₃)₃, 47.3 (CH₂ ring), 49.8 (CH₂ ring), 51.3 (CH₂ ring), 53.7 (CH₂ ring), 55.0 (NCH₂CO), 66.1 (OCH₂Ph), 79.1 (C(CH₃)₃), 79.4 (C(CH₃)₃), 128.4 (Ph), 128.5 (Ph), 135.6 (Ph), 155.2 (CONH), 155.7 (CONH), 155.9 (CONH), 170.3 (COO). FTIR (KBr): 3010, 2979, 2932, 2448, 1689, 1464, 1417, 1363, 1243, 1167, 1025, 982, 912 cm⁻¹. *m/z* (ESI+) ([M + H]⁺ 621, [M + Na]⁺ 643, [M + K]⁺ 659). Anal. Found C = 60.7%, H = 8.4%, N = 8.8% C₃₂H₅₂N₄O₈ · H₂O. Theory: C = 60.6%, H = 8.8%, N = 8.8%.

1,4,7,10-Tetraazacyclododecane-1-benzyl Acetate Trihydrochloride Salt (5)

HCl (12 M, 5 mL) was added dropwise to a solution of **4** (5.0 g, 8.1 mmol) in ethanol (10 mL). The resulting solution was stirred for 3 h at room temperature. The reaction mixture was concentrated to 5–6 mL and the precipitates were filtered. The residue was crystallized from a mixture of water and tetrahydrofuran to afford a colorless crystalline compound (3.0 g, 81% yield). ¹H NMR (270 MHz, D₂O, (pD = 2): δ = 2.9 (16H, s br, ring CH₂), 3.6 (2H, s, CH₂COO), 5.2 (2H, s, OCH₂Ph), 7.4 (5H, m, Ph). ¹³C NMR (67.5 MHz, D₂O, dD = 2) δ = 41.7 (CH₂ ring), 42.3 (CH₂ ring), 44.3 (CH₂ ring), 49.4 (CH₂ ring), 54.1 (NCH₂CO), 68.0 (OCH₂Ph), 128.9 (Ph), 129.2 (Ph), 135.3 (Ph), 173.3 (CO₂). FTIR (KBr): 3398, 2974, 2645, 2435, 1743, 1611, 1565, 1480, 1460, 1440, 1375, 1351, 1277, 1188, 1167, 1070, 940 cm⁻¹. *m/z* (ESI+) 321 (100% [M + H]⁺). Anal. Found C = 45.5%, H = 7.7%, N = 12.9% C₁₇H₂₈N₄O₂ · 3HCl · H₂O. Theory: C = 45.6%, H = 7.4%, N = 12.5%.

1,4,7,10-Tetraazacyclododecane-1,4,7-tris(methylcarbonylamide), 10-Benzyl Acetate (6)

K₂CO₃ (9.0 g, 65 mmol) was added to monoprotected cyclen **5** (2.9 g, 6.5 mmol) in acetonitrile (50 mL). The suspension was heated for 20 min at 60 °C. 2-Bromo-*N*-methylacetamide (2.96 g, 19.6 mmol) was then added in one portion. The reaction mixture was heated overnight at 60 °C then cooled, filtered, and washed with cold acetonitrile (2 × 50 mL). The solids were dissolved in DI water (30 mL), extracted with dichloromethane (3 × 250 mL), and the combined organic fractions dried over Na₂SO₄. The solvents were removed under reduced pressure to afford a colorless product (3.29 g, 95% yield). ¹H NMR (270 MHz, CDCl₃): δ = 2.1–2.6 (16H, m br, CH₂ ring), 2.7 (3H, s, CH₃), 2.75 (6H, s, CH₃), 3.11–3.17 (8H, m, CH₂CO), 5.1 (2H, s, OCH₂Ph), 7.3 (5H, m, Ph), 7.8 (4H, s br, NHCH₃). ¹³C NMR (67.5 MHz, CDCl₃) δ = 26.1 (CH₃), 26.2 (CH₃), 50.5 (CH₂ ring), 51.5–51.7 (CH₂ ring), 55.2 (CH₂CONH), 57.2 (CH₂CONH), 57.6 (CH₂CO), 67.0 (CH₂Ph), 128.5 (Ph), 128.7 (Ph), 135.5 (Ph), 171.6 (CONH), 171.9 (CONH), 172.4 (COO). FTIR (KBr): 3258, 3076, 2970, 2948, 2823, 1732, 1654, 1553, 1449, 1410, 1370, 1304, 1235, 1192, 1103, 990 cm⁻¹. *m/z* (ESI+) 556 (100% [M + Na]⁺); Anal. Found C = 46.8%, H = 8.5%, N = 15.0% C₂₆H₄₃N₇O₅ · 7.5 H₂O. Theory: C = 46.7%, H = 8.8%, N = 14.7%.

1,4,7,10-Tetraazacyclododecane-1,4,7-tris(methylcarbonylamide)-10-acetate (1)

10 % palladium on carbon (0.8 g) was added to a solution of **5** (4.3 g, 8.1 mmol) in ethanol (30 mL). The reaction mixture was placed in a hydrogenation vessel for 24 h with the H₂ pressure of 45 psi. The catalyst was filtered through filter agent (Celite545, Aldrich) and the

solvents were removed under reduced pressure to afford a colorless solid (2.9 g, 81% yield). ^1H NMR (270 MHz, D_2O): δ = 2.7 (6 H, s, CH_3), 2.8 (3H, s, CH_3), 3.0 (4H, s br, ring CH_2), 3.18 (4H, s br, ring CH_2), 3.4 (8H, s br, ring CH_2), 3.5 (4H, s, CH_2CONH), 3.8 (2H, s, CH_2CONH), 4.0 (2H, s, CH_2CO_2). ^{13}C NMR (67.5 MHz, D_2O): δ = 26.2 (CH_3), 26.3 (CH_3), 48.1–51.5 (CH_2 ring, br), 51.9 (CH_2CONH), 54.6 (CH_2CONH), 56.1 (CH_2CO_2), 166.2 (CONH), 169.9 (CONH), 172.4 (CO_2). FTIR (KBr): 3262, 3095, 2967, 2843, 1654, 1561, 1452, 1394, 1157, 1087, 1046, 909 cm^{-1} . m/z (ESI $^-$) 442 (100% $[\text{M} - \text{H}]^-$).

Preparation of Thulium Complexes

An aqueous solution of the ligand **L2** was adjusted to pH = 5.5 using 1 M NaOH. A stoichiometric quantity of a standardized aqueous thulium chloride solution was added and the reaction mixture stirred at room temperature with constant adjustment of pH to 5.0–5.5 using 1 M NaOH. After approximately 1 h, no further drop in pH was observed and the final pH was adjusted to 7 followed by filtration. The absence of free thulium was verified by adding a small aliquot of this equilibrium mixture to a solution of 0.15 M acetate buffer, pH 5.5, containing a drop of xylenol orange as indicator. Once verified, the **TmL2** solution was freeze-dried and stored in powdered form at -20°C . **Tm-L2**: FTIR (KBr): 2120 ($\text{N}=\text{C}=\text{S}$) cm^{-1} ; m/z (FAB $^+$) 770 $[\text{Tm}^{3+}\text{L2} - 2\text{H}]^+$.

1,4,7,10-Tetraazacyclododecane-1,4,7-tris(methylcarbonylamide)-10-1-*N*-hydroxy Succinimidyl Acetate (7)

Acetate (**7**) was obtained by the method of Lewis et al. (19). Compound **L1** (2.9 mg, 6.6 μmol) was dissolved in water (1.043 mL, 4°C), and the pH of the solution adjusted to 5 with 15 μL of 1 M HCl. *N*-Hydroxysuccinimide (0.76 mg, 6.6 μmol) was dissolved in water (0.06 mL, 4°C) and added. The reaction mixture was stirred for 5 min, followed by addition of 0.05 mL solution of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (0.63 mg, 3.3 μmol) at 4°C . The reaction mixture was stirred for 30 min on ice (at 0°C) and the final pH of the reaction mixture adjusted to 7.4 by addition of 50 μL of 0.2 M Na_2HPO_4 . The active ester was used for modification of adenovirus particles without further purification.

Cell Lines

Cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS). Standard cell culture conditions were incubation at 37°C and 5% CO_2 . The 911 cell line used for propagation of recombinant adenovirus is derived from a human embryonic retinoblast and contains an integrated copy of the leftmost portion of the adenovirus 5 genome that complements the defect in E1 replacement vectors. The HT1080 cells used for *in vitro* bioactivity determination of viral particles are a human fibrosarcoma tumor cell line.

Preparation of Purified Recombinant Adenovirus

The AdCMVLuc virus (20) used here was chosen for ease of bioactivity analysis. 911 cells were cultured in DMEM containing 10% FBS grown to near 100% confluency. The growth medium was removed, and cells were infected by adding virus stock solution diluted into 2.5 mL DMEM, 2% FBS. The plate was rocked at 15 min intervals for 1 h. DMEM, 2% FBS (15 mL) was added to the dish, followed by incubation at 37°C for 3–5 days. The cell were lysed by addition of 10% Nonidet P-40 (US Biochemical), and the cell lysate was centrifuged at 10 000 rpm for 10 min at 4°C in a Sorvall GSA rotor to separate the cell debris. The supernatant was saved and one-half volume of 20% PEG 8000, 2.5 M NaCl, was added to each bottle. The bottles were incubated on ice for 1 h to precipitate the virus particles. The precipitated virus was collected by centrifugation at 10 000 rpm for 10 min at 4°C . The viral pellet was resuspended in 5 mL of CsCl ($d = 1.10 \text{ g/mL}$) in 20 mM Tris HCl pH 8.0 buffer. The suspension

was centrifuged for 5 min at 8000 g at 4 °C in a Sorval SS-34 rotor. CsCl gradients were prepared as follows: 2.0 mL CsCl ($d = 1.40$ g/mL) in 20 mM Tris HCl (pH 8.0) buffer on the bottom of a centrifuge tube, and 3.0 mL CsCl ($d = 1.30$ g/mL) in 20 mM Tris HCl (pH 8.0) buffer on the top of the previous layer. The viral CsCl supernatant in 20 mM Tris HCl (pH 8.0) buffer was layered on the top of the CsCl step gradient and centrifuged at 20 000 rpm for 2 h at 20 °C in a Beckman SW41 swinging bucket ultracentrifuge rotor. The white viral opalescent band, which appeared as a band between the 1.3/1.4 CsCl layers, was collected. Virus was further purified by gel permeation chromatography using Sepharose CL-4B equilibrated with isotonic phosphate (PBS, 137 mM NaCl, 2.74 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.5) or carbonate (0.05 M Na₂CO₃/NaHCO₃, 140 mM NaCl, pH 8.6) buffers. Virus was stored at -80 °C after addition of glycerol to 10% final concentration until use.

Conjugation of L1 to Adenovirus

To a 0.1 mL aliquot of adenovirus particles (1.0×10^{13} particles/mL, 1.66 pmol) in PBS buffer was added a solution of active ester **7** in phosphate buffer (2.7 mM) at different molar ratios. Additional buffer was added as necessary to keep the volume in all vials constant. The reaction mixture was incubated at 4 °C for 4 h on the rocker. Spin columns were prepared for gel permeation chromatography as follows: a 3 mL disposable syringe was plugged with cotton and filled with Sepharose CL-4B equilibrated in acetate buffer (0.15 M CH₃COONH₄, 0.14 M NaCl, pH 7). Those columns were washed by centrifugation at 3000 rpm for 3 min at least 5 times until a constant volume of eluent was obtained prior to conjugate purification. Each reaction mixture was placed on a spin column and centrifuged at 3000 rpm for 3 min in an Eppendorf Centrifuge model 5810 R. After viral loading, the spin column was washed once with 0.2 mL acetate buffer. The two fractions were collected, vortexed, centrifuged, and the absorbance at 260 nm was measured. The samples were analyzed to determine the ligand/adenovirus ratio and bioactivity.

Conjugation of L2 to Adenovirus

L2 (5 mg, 8.3 μ mol) was dissolved in water (0.1 mL), followed by addition of 2.86 mL of carbonate buffer previously cooled to 4 °C. To 0.2 mL of adenovirus (1×10^{13} particles/mL, 3.32 pmol) in carbonate buffer (0.05 M Na₂CO₃/NaHCO₃, 140 mM NaCl, pH 7.9) were added different amounts of **L2**. The volume of each reaction mixture was adjusted to keep it constant in all vials. The reaction mixtures were placed in a cooled room (4 °C) on the continuous mixing rocker for 6 h. The **L2**-AdCMVLuc conjugates were purified in a manner identical to that of **L1**-AdCMVLuc.

Kinetics of L1 and L2 Conjugation to Adenovirus Particles

To 0.4 mL of adenovirus in PBS (4×10^{12} particles/mL) or carbonate (5×10^{12} particles/mL) buffer was added 61 μ L (2.71 mM) of freshly prepared active ester **7** or 49 μ L (2.8 mM) of isothiocyanate (**L2**). The reaction mixtures were incubated at 4 °C on the continuous mixing rocker, and at different time intervals 100 μ L of each reaction mixture was taken and purified to evaluate the ligand/adenovirus ratio and bioactivity.

Determination of Ligand/Adenovirus Ratio

The number of ligands per adenovirus particle was determined by using Pb(II) as a colorimetric indicator as previously described (21). Briefly, the method is based on the decrease in absorbance at 656 nm that occurs upon transchelation of Pb²⁺ from arsenazo III to the virus-bound chelate sites. Experimentally, 100 μ L of each conjugate solution was incubated with Pb(II)-Arzenazo III for 30 min to allow sufficient time for binding of Pb²⁺ into all chelate sites on the virus (21) prior to measuring the absorbance at 656 nm. A calibration curve was established by mixing known concentrations of the parent ligand **L1** or **L2** (0.0–2.5 μ M) with

the lead reagent and measuring the absorbance at 656 nm. The virus concentration was obtained by measuring absorbance at 260 nm (1 OD unit corresponds to 1×10^{12} particles/mL).

Cell Infection and Bioactivity

The bioactivity of the viral preparations and viral conjugates was assessed by infection of HT1080 cells with an adenovirus vector that expresses firefly luciferase from the cytomegalovirus (CMV) promoter. The expression of luciferase activity in the cells infected with adenovirus is directly proportional to the number of infecting virus particles. HT1080 cells were cultured in DMEM with 10% FBS in 12 well plates. The growth medium was removed and 0.5 mL fresh (warmed to 37 °C) medium was added to each well. Aliquots (1, 3, and 10 μ L) of a purified adenovirus conjugate or control unmodified adenovirus were added to wells in duplicate. After a 1 h incubation period at 37 °C to allow the virus to bind and infect cells, the infection medium was removed and replaced by 1 mL of fresh medium (warmed to 37 °C). The plates were then incubated for 15–19 h at 37 °C to allow luciferase expression (22).

Luciferase Assay

Infected HT1080 cells were assayed for luciferase expression as previously described (14). Briefly, cells were lysed by addition of 0.5 mL of lysis buffer (25 mM Tris–phosphate buffer pH 7.8, 2 mM dithiothreitol (DDT), 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid 10% glycerol, 1% Triton X-100). Extracts (10 μ L) were mixed with 0.1 mL of lysis buffer, and to that 50 μ L of luciferin reagent (20 mM tricine, pH 7.8, 1.07 mM (MgCO_3) $\times$ 4Mg (OH)₂, 2.67 mM MgSO_4 , 0.1 mM EDTA, 33.3 mM DTT, 270 μ M coenzyme A, 470 μ M luciferin, 530 μ M ATP) was added. Luminescence was measured for 10 s and expressed in relative light units (RLU).

Adenovirus Stability as a Function of Time

To 0.2 mL adenovirus (4×10^{12} particles/mL) in PBS (pH 7.4) or carbonate buffer (pH 7.9) was added 150 μ L of PBS (pH 7.4) or carbonate buffer (pH 8.6) and viruses were incubated in the cold room at 4 °C. At different time intervals, aliquots were taken and samples used to infect HT1080 cells in duplicate. After 24 h, cells were extracted and assayed for luciferase expression as a measure of viral infectivity.

¹⁷⁷Lu-Adenovirus Binding Assays

The amount of complexed versus free ¹⁷⁷Lu³⁺ was determined by radio-TLC. 0.5 μ L sample of a virus or virus-conjugate solution was spotted onto silica gel 60 TLC plates (EDM Chemicals Inc.) and, after drying with a stream of cold air, eluted with 10% ammonium acetate in methanol (1:3 v/v). Prior to TLC, excess DTPA was added to each virus sample to quantify the amount of radioactivity not associated with the adenovirus. As free uncomplexed ¹⁷⁷Lu (Ac)₃ and ¹⁷⁷Lu³⁺-conjugated virus particles remain at the origin while ¹⁷⁷LuDTPA²⁻ moves with the solvent front, it was easy to distinguish ¹⁷⁷Lu³⁺-conjugated virus and ¹⁷⁷Lu³⁺ associated with the virus nonspecifically (R_f = 0–0.12) from ¹⁷⁷LuDTPA²⁻, which migrated with the solvent front (R_f = 0.48–0.50).

The virus conjugates were loaded with ¹⁷⁷Lu³⁺ as follows: 5 μ L of ¹⁷⁷Lu³⁺ in 0.05 N HCl (11–12 μ Ci) was mixed with 15 μ L of acetate buffer (0.15 M ammonium acetate, pH 8) then added to 50 μ L of adenovirus (1.15×10^{12} particles/mL) in acetate buffer (0.15 M ammonium acetate, 140 mM NaCl). The reaction mixture was incubated at 25 °C for various selected time intervals, then 10 μ L of the mixture was challenged with 20 000-fold excess DTPA (10 mM, pH 8). After an equilibration period of 30 min, the samples were analyzed by radio-TLC.

Preparation of TmL2-Adenovirus Conjugates

Adenovirus (2.4 mL, 5×10^{12} particles/mL) in Tris buffer was placed on a Sepharose CL-4B column equilibrated with HEPES buffer (pH 8.6 10 mM HEPES, 135 mM NaCl, 7 mM KCl). Adenovirus (3.1 mL, 3.85×10^{12} part/mL) was mixed with 355 μ L of TmL2 (2.8 mM) in HEPES buffer (pH 8.6). The reaction mixture was incubated in a cold room at 5 °C with continuous stirring for 3 h, followed by quenching with 1 mL of 10 mM Tris HCl buffer pH 7.4. The adenovirus conjugate was concentrated by CsCl gradient centrifugation (on a discontinuous CsCl gradient of 1.3 and 1.4 g/mL CsCl as before) and gel filtered on a Sepharose CL-4B column equilibrated with HEPES buffer (pH 7.4, 10 mM HEPES, 135 mM NaCl, 7 mM KCl).

RESULTS AND DISCUSSION

Synthesis

L1 was synthesized as outlined in Scheme 2. Tris-Boc-protected cyclen **3** was obtained by published methods (16) followed by monoalkylation of the free amine with benzyloxy-bromoacetate under standard conditions (K_2CO_3 /MeCN). Excess alkylating agent was removed by column chromatography to yield intermediate **4** followed by smooth removal of the Boc groups using an ethanolic hydrochloric acid to generate monobenzyl protected cyclen **5** as the HCl salt. The free amines of **5** were alkylated with 2-bromo-*N*-methylacetamide and 10 equiv of K_2CO_3 to produce **6**. It was not necessary to isolate the monobenzyl protected cyclen **5** in the free base form to perform this reaction. Compound **6** was conveniently isolated in analytically pure form by precipitation from acetonitrile. **L1** was obtained by removal of benzyl protective group by hydrogenolysis with H_2 and 10% palladium on carbon in ethanol. The *N*-hydroxysuccinimide ester of **L1** was generated *in situ* according to a previously published method (19). Ligand **2** was synthesized using a previously published method (17).

Conjugation of L2 to Adenovirus

The isothiocyanate of DOTA-tetraamide reacts with the ϵ -amino group of viral surface lysine residues to produce a thiourea linkage under basic conditions (Scheme 3). The method of Mirzadeh et al. (23) was adapted to generate the **L2**-adenovirus conjugates. To estimate the number of reactive surface lysine residues per adenoviral particle, a set of reactions were run where the amount of added ligand **L2** was varied while the virus concentration was kept constant. Ligand **2** was reacted with adenovirus at [**L2**]: adenovirus ratios ranging from 10^3 :1 to 10^6 :1. Unreacted bifunctional chelator was removed from the viral conjugates by gel permeation chromatography, which removed >99% of the unreacted bifunctional agent as indicated by ^{177}Lu radiolabeling studies. Incubation of each virus conjugate with $^{177}Lu^{3+}$ and consequent analysis by radio-TLC showed that most of the radioactivity was associated with a fraction having an R_f of ~0.1 The number of chelates on the adenovirus surface was established using a published Pb^{2+} -arsenazo III colorimetric method (21) and the adenovirus concentration was obtained by UV absorbance. Figure 1 shows that the reaction conditions described above yielded virus particles with up to ~2000 **L2** chelates per adenovirus particle.

Conjugation of L1 to Adenovirus

The **L1** activated ester (compound **7**) was used to react with surface lysine residues of the adenovirus particles to form amide linkages (Scheme 3). Since the **L1** active ester was not isolated prior to its reaction with virus, the theoretical concentration of bifunctional agent is based on the concentration of EDC as the limiting reagent. To establish the maximum loading number of modified lysine residues with **7**, a similar strategy as described above for ligand **L2** was undertaken. However, due to complete loss in adenovirus bioactivity when using a 5×10^5 :1 ratio of **L2**: AdCMVLuc, the reaction stoichiometry was reduced to **7**:AdCMVLuc

ratios of $10^3:1$, $5 \times 10^4:1$, $10^4:1$, $10^5:1$, and $5 \times 10^5:1$. The reaction mixtures were purified by gel permeation chromatography and analyzed by radio-TLC showing >99% radioactivity associated with the fraction having $R_f = 0.07-0.11$. Figure 1 shows that the number of attached chelates gradually increased to a maximum of ~1100 with increasing concentrations of the active ester, **7**. A comparison of the two conjugation experiments shows that the number of attached **L1** ligands maximizes near ~1100 chelators with no significant increase in the number of chelators observed beyond this point, whereas the number of attached **L2** ligands approached a maximum of ~2000. This difference most likely reflects the tendency of the more reactive NHS ester to hydrolyze under these experimental conditions, although the reduced steric demand of **L2** relative to **L1** may also play a role at the higher conjugation efficiency of **L2**.

Optimal Reaction Times for Lower Concentrations of Reactive Bifunctional Agents

In a second series of experiments, the active ester of **L1** (ligand **7**) or **L2** was added to adenovirus in 50 000-fold excess at 4 °C, and after various incubation periods (Figure 2), the reactions were terminated by rapid gel permeation chromatography using spin columns. Although the active ester **7** reacts more rapidly with lysine amino groups initially (Figure 2), after 2 h there was no significant difference in the total number of **L1** versus **L2** ligands attached likely due to competitive hydrolysis of NHS ester at this reaction pH. In separate experiments, it was shown that the active ester **7** was consumed within 2 h. These data show that both reagents modify an equal number of surface lysine groups (~1200) after a 2 h incubation time at 4 °C.

Bioactivity (Luciferase) Assay

In order to be considered useful as a vector for optical imaging, the modified virus must maintain an ability to infect cells and express luciferase. To evaluate the effects of chemical modification, viral bioactivity was assessed by infecting HT 1080 cells with modified adenovirus particles. As shown in Figure 3, a significant decline in viral bioactivity was observed upon modification of surface lysines with either reactive ligand **L1** or **L2**. Viral bioactivity decreased almost linearly with increasing number of conjugated ligands until about 800 ligands were attached. When the number of attached ligands reached ~1000 per viral particle, viral infectivity dropped precipitously, as these conjugation reactions derivatize not only reactive lysine groups on the hexon capsid proteins of the core viral particle but also the fiber proteins which are responsible for the attachment of the virus to the host cell. The results shown in Figure 3 suggest that 800–900 sites can be modified without a dramatic loss in bioactivity, but further modification has a much more dramatic effect on the ability of the virus to infect cells. In samples where ~1100 molecules of **L1** or 1200 molecules of **L2** were conjugated, viral bioactivity decreased to ~20% of control values (20% residual activity). This amount of residual activity could reflect a subpopulation of particles that are substituted at a fractionally lower level than the average particle in the population, but there is little reason to assume such an anisotropic distribution in the extent of substitution across the population of particles given that there are hundreds to thousands of potential substitutions per particle, and a trillion particles per milliliter in the solution. We assume that average level of substitution is reflective of the average level of bioactivity, some with more substitution per particle (and less infectivity), some with less (and more infectivity). For the purposes of this study, a goal of creating labeled virus particles with a minimum of 50% bioactivity was considered acceptable.

To achieve this target, another set of experiments was performed where the amount of excess **L1** or **L2** was lowered to 50 000-fold excess and aliquots of sample were removed as a function of time to follow the progression of the chemical reactions. Those results are reported in Figure 4. After a 1 h conjugation reaction using the active ester **7**, 790 chelators were attached and at the same time ~70% of bioactivity was retained. In comparison, the isothiocyanate, **L2**, showed 730 chelators were loaded per adenoviral particle after a 1.5 h incubation time and this virus maintained ~72% bioactivity. This indicates that, when using an initial excess of 50 000

bifunctional chelators per virus particle, the conjugation reaction should be terminated at 1 and 1.5 h for **L1** and **L2**, respectively, to achieve optimal results. Any potential loss of bioactivity arising from instability of the virus in PBS or carbonate buffer was eliminated by running a set of control reactions. Indeed, the adenovirus maintained a full ability to infect cells upon incubation in PBS (pH 7.4) and carbonate (pH 7.9) for 9 h at 4 °C (Figure 4).

Radiolabeling of Adenovirus

Initial attempts to fill only the conjugated ligand sites on the surface of the adenovirus with either Eu^{3+} or $^{177}\text{Lu}^{3+}$ proved unsuccessful because of nonspecific binding of these metal ions at other virus particle locations. To better understand this initial observation, unmodified adenovirus particles were incubated with $^{177}\text{Lu}^{3+}$ and then challenged with an excess of DTPA to quantify the amount of radioactivity associated with the viral particle through nonspecific binding. Figure 5 shows that the amount of nonspecific binding of $^{177}\text{Lu}^{3+}$ to the virus particles increases with incubation time and maximizes at ~10% after 9 h. This shows that $^{177}\text{Lu}^{3+}$ gets trapped by the viral particle, perhaps internally, by binding sites that are inaccessible to DTPA. Similar observations were made by Basu et al. (24), where they identified 180 metal binding sites based on the crystal structure of cowpea chlorotic mottle virus. Each binding site is thought to be composed of five amino acid residues from two adjacent protein subunits. Tb^{3+} binding, as measured by fluorescence, was tested both on the wild-type virus and a mutant virus where the RNA had been removed. In both cases, metal binding displayed very similar K_d values ($K_d = 19 \mu\text{M}$ and $17 \mu\text{M}$, respectively) suggesting that the encapsidated RNA does not contribute to the metal binding. Even though adenovirus represents a very different class of virus, it is tempting to speculate that the DNA in its interior does not bind metal ions nonspecifically. Heavy metal binding sites have been identified using the crystal structure of hexon, the major coat protein of adenovirus (25). These sites are located close to the loops l_1 , l_2 , or l_3 on the surface of the protein molecule or l_4 which lies underneath the hexon. The loop l_4 of each hexon protein might be particularly inaccessible to DTPA. Since this nonspecific binding of heavy metals might complicate the interpretation of any subsequent imaging experiments, an alternative prelabeling approach was adopted for the **L2** system where the ligand itself could be preloaded with the desired metal ion prior to labeling the virus. This strategy could not be used for the more reactive activated ester form of **L1**.

Conjugation of TmL2 to Virus Particles

Given that the detection limit of thulium-based PARACEST agents has been reported to be in the micromolar range (26), adenovirus particles were labeled with the prelabeled reagent, TmL2, to evaluate whether CEST can be detected from samples of surface-labeled virus particles. The TmL2-adenovirus conjugate was prepared using techniques similar to that of the parent ligand (Scheme 3). Two control experiments were run; first, an unmodified adenovirus was prepared in HEPES buffer and incubated with TmDTMA $^{3+}$ (1,4,7,10-tetra(methylcarbonylamide)-1,4,7,10-tetraaza cyclododecane), a nonbifunctional version of TmL2. This control was performed to estimate the effect of entrapment or strong electrostatic interactions of positively charged complexes with viral particles. The control samples were treated in a similar fashion to that of TmL2-adenovirus conjugates. The concentrations of the viral particles were adjusted to maintain a constant 2.5×10^{13} particles/mL, and the thulium concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS). The TmL2-adenovirus conjugates were found to contain 1140 thulium ions per adenovirus particle, indicating that a result similar to conjugation with nonprelabeled **L2** was obtained (Figure 3). Somewhat unexpectedly, adenovirus particles incubated in the presence of TmDTMA $^{3+}$ for 3 h and consequently purified also contained ~280 entrapped TmDTMA $^{3+}$ complexes. This indicates that the tripositive complex, TmDTMA $^{3+}$, is tightly bound by virus particles and is not removed by simple gel purification procedures. Even though the stability of TmDTMA $^{3+}$ ($\log K_{\text{TmL}} = 14.08$) is considerably lower than the parent chelate,

TmDOTA⁻, it has been shown that other lanthanide cations (Ln³⁺) dissociate much more slowly from tetraamide ligands such as DTMA, **L1**, or **L2** than from DOTA (26), so the possibility of Tm³⁺ release from any of these chelates on the adenovirus particle is unlikely. Thus, we feel confident that thulium, as measured by ICP-MS for both the control and conjugation products, reflects Tm-DTMA³⁺ and Tm**L2** and not free Tm³⁺ released from these chelates. The results of the control and radiolabeling experiments show that a significant level of nonspecific binding by any positively charged species, whether it be a simple cation such as ¹⁷⁷Lu³⁺ or a chelated cation such as TmDTMA³⁺, is difficult to avoid.

CEST Studies

Recently, Vinogradov et al. (27) reported that Tm³⁺-based PARACEST agents can be detected by imaging in the μ M (micromolar) range by using a modified on-resonance low-power WALTZ-16 pulse train for activation. To apply this technique to labeled virus particles, the intensity of the solvent–water signal was measured after application of WALTZ-16 pulse train directly on the water resonance and compared to a spectrum collected by placing the WALTZ-16 pulse train far off resonance. Any differences in intensities between those two spectra should then reflect rapid water exchange on and off the viral-bound Tm³⁺ PARACEST agent (27). The modified WALTZ-16 pulse sequence depends on the number of parameters including T_1 and T_2 relaxation times of bulk water and the lifetime of water molecule coordinated to thulium ion in the complex (τ_M). Considering the relatively short bulk water transverse relaxation time (T_2 , Table 1) of the adenovirus solution (~ 0.22 ms at [virus] = $(2.5 \pm 0.1) \times 10^{13}$ particles/ mL), a WALTZ pulse length of 856 μ s and irradiation power of 291 Hz was chosen (27).

The solvent–water T_1 relaxation times for these samples were on the order of 2 s, a value considered adequate for this type of WALTZ-16 experiment (27). The experimental T_2 values were however considerably shorter, in the range 0.12–0.24 s, and as predicted by theory, the biggest effect on the magnetization will occur whenever T_2 is ~ 0.4 s. On the basis of those predictions and to subtract out the influence of T_2 , the WALTZ-16 experiment was performed first on an unmodified adenovirus sample (as control) and the Tm**L2**-adenovirus sample. As shown in Figure 6, the water intensity was reduced by $\sim 20\%$ for the unmodified virus sample (Figure 6b) and 18% for adenovirus incubated in the presence of TmDTMA³⁺ (Figure 6c), compared to buffer alone. Although there was considerable entrapment of TmDTMA³⁺ (noncovalent interactions) when incubated with the adenovirus, the presence of this compound did not change M_Z/M_0 compared to unmodified virus. This suggests that the entrapped TmDTMA³⁺ is not exchanging protons rapidly with bulk water and furthermore shows that the adenovirus alone causes a decrease in water intensity using the WALTZ-16 pulse train due to its short T_2 . The T_2 of the adenovirus–TmDTMA³⁺ sample was somewhat shorter than adenovirus alone, likely due to the presence of ~ 280 entrapped TmDTMA³⁺ complexes per adenovirus particle. Interestingly, a substantially larger effect was measured for the Tm**L2** - adenovirus conjugate, in which a 32% decrease in solvent–water intensity was observed. The 12% difference in water intensity between these two samples can be directly attributed to the presence of the conjugated PARACEST agent on the surface of the adenovirus.

The dependence of TmDTMA³⁺ concentration on the reduction of observed magnetization (M_Z/M_0) using WALTZ 16 pulse train was also investigated as a positive control. Figure 7 summarizes the results and clearly shows that 48 μ M Tm-DTMA³⁺ (equivalent to the concentration of Tm**L2** in the virus sample used in Figure 6) produces a modest 2% reduction in signal intensity. Thus, 12% difference in water intensity observed for the Tm**L2** virus particle is not simply due to the presence of conjugated Tm**L2** in solution, but must reflect some exchange advantage of viral-conjugated Tm**L2** compared to the nonconjugated parent complex, TmDTMA³⁺. This suggests that water exchange in Tm**L2** is altered when bound to

the surface of virus particles (compared to that seen for TmDTMA³⁺) and this change is advantageous for CEST. Whether this reflects an increased or decreased water exchange rate for the viral-bound TmL2 (both could result in a CEST advantage if operating in certain water exchange regimes (28)) will require more detailed studies that are beyond the scope of the present investigation.

CONCLUSIONS

Labeling of virus particles with two different bifunctional ligands resulted in diminished viral bioactivity that decreased in proportion to the number of the attached ligands. For in vivo applications where it is desirable to have at least 50% viral bioactivity, it was found that the number of attached ligands needed to be fewer than ~800 to maintain 75–80% bioactivity. Labeling experiments with ¹⁷⁷Lu³⁺ and TmDTMA³⁺ at 4 °C (as a free chelate control) showed nonspecific binding of positively charged species to the adenovirus. To circumvent nonspecific metal ion binding to the adenovirus, the isothiocyanate bifunctional ligand (L2) was preloaded with Tm³⁺ and the resulting chelate was used to label the adenovirus with a conjugation efficiency similar to that of free L2. The resulting TmL2-adenovirus conjugate showed a 12% decrease of water signal intensity using a WALTZ-16 sequence for CEST activation. These results demonstrate that PARACEST-labeled virus particles can be prepared that retain high viral infectivity and expression of luciferase activity and, at the same time, are sufficiently sensitive to be detected by WALTZ-16 initiated CEST imaging.

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Figure 1.

Plot showing the number of ligands attached per virus particle as a function of initial ligand concentration. Ligands **L1** and **L2** were incubated at 4 °C for 4 h (**L1**) and 6 h (**L2**) to allow for virus modification.

Figure 2. Plot showing the number of ligands attached per virus particle as a function of incubation time for ligands **L1** and **L2**. All modifications were performed at 4 °C.

Figure 3.

Plot showing the reduction in viral infectivity as a function of the number of ligands attached to virus particles. At each time point shown in Figure 2, an aliquot of modified virus was purified and used to infect HT1080 cells. Unmodified virus was used as a control and the extent of luciferase expression (in RLU) was set to 100%. Luciferase activity measured in each modified virus sample was expressed as a percentage of control.

Figure 4.

Plot showing the loss in bioactivity as a function of time after addition of 50 000-fold excess ligand (either **L1** or **L2**) to viral particles.. The initial time points (0 h) correspond to the bioactivity of unmodified adenovirus in the corresponding buffer . Control represents the ability of unmodified adenovirus to infect HT1080 cells after incubation at 4 °C.

Figure 5. Residual $^{177}\text{Lu}^{3+}$ bound to adenovirus particles as a function of incubation time. At the indicated time-points, 20 000-fold excess of DTPA (pH 8) was added to remove weakly bound $^{177}\text{Lu}^{3+}$ and the virus was purified by gel filtration chromatography and counted

Figure 6.

Residual bulk water signal intensities after applying WALTZ-16 pulse train ($B_0 = 500$ MHz, $B_1 = 291$ Hz, pulse length 0.856 ms, 25 °C) either “off” resonance where the carrier frequency is set to 1986 Hz away from the water resonance or “on” resonance where the carrier frequency is set at the water frequency. (A) corresponds to a sample containing TmL2-adenovirus in the HEPES buffer (10 mM HEPES, 135 mM NaCl, 7 mM KCl); the total concentration of TmL2 in the sample was 48 μ M. (B) 48 μ M TmDTMA³⁺ in HEPES buffer. (C) an equivalent amount of unmodified adenovirus (as in A) in HEPES buffer.

Figure 7.

Plot of the decrease in water magnetization (M_z/M_0) using the WALTZ-16 pulse sequence as a function of TmDTMA³⁺ concentration. The WALTZ-16 pulse train was applied on resonance ($B_0 = 500$ MHz, $B_1 = 291$ Hz, pulse length 0.856 ms, 25 °C).

L1 **L2**

Scheme 1.

Scheme 2.

Scheme 3.

Table 1Experimental T_2 Relaxation Times and M_Z/M_0 for Adenovirus Particles

	T_2 , s	M_Z/M_0 , %	[Tm ³⁺], μM	[virus], 10^{-7} M
adenovirus	0.244 ± 0.002	80	N/D	0.42
adenovirus-TmDTMA	0.220 ± 0.002	82	11.8	0.42
adenovirus-TmL2	0.127 ± 0.002	68	48	0.42