Published in final edited form as:

Bioconjug Chem. 2007 ; 18(6): 2061–2067. doi:10.1021/bc070075t.

Enhanced Retention of the α -particle Emitting Daughters of Actinium-225 by Liposome Carriers

Sofou Stavroula^{‡,*†}, Barry J. Kappel[‡], Jaspreet S. Jaggi[‡], Michael R. McDevitt[‡], David A. Scheinberg[‡], and George Sgouros[§]

[‡] Molecular Pharmacology and Chemistry, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021

[§] Department of Radiology, Division of Nuclear Medicine, Johns Hopkins Medicine, 4M61 Cancer Research Building II, 1550 Orleans St, Baltimore, MD 21231

Abstract

Targeted α -particle emitters hold great promise as therapeutics for micrometastatic disease. Because of their high energy deposition and short range, tumor targeted α -particles can result in high cancer-cell killing with minimal normal-tissue irradiation. Actinium-225 is a potential generator for α -particle therapy: it decays with a 10-day half-life and generates three α -particle emitting daughters. Retention of ^{225}Ac daughters at the target increases efficacy; escape and distribution throughout the body increases toxicity. During circulation, molecular carriers conjugated to ^{225}Ac cannot retain any of the daughters. We previously proposed liposomal encapsulation of ^{225}Ac to retain the daughters, whose retention was shown to be liposome-size dependent. However, daughter retention was lower than expected: 22% of theoretical maximum decreasing to 14%, partially due to binding of ^{225}Ac to the phospholipid membrane. In this study, MULtiVEsicular Liposomes (MUVELs) composed of different phospholipids were developed to increase daughter retention. MUVELs are large liposomes with entrapped smaller lipid-vesicles containing ^{225}Ac . PEGylated MUVELs stably retained over time 98% of encapsulated ^{225}Ac . Retention of ^{213}Bi , the last daughter, was 31% of the theoretical maximum retention of ^{213}Bi for the liposome sizes studied. MUVELs were conjugated to an anti-HER2/neu antibody (immunolabeled MUVELs), and were evaluated *in vitro* with SKOV3-NMP2 ovarian cancer cells, exhibiting significant cellular internalization (83%). This work demonstrates that immunolabeled MUVELs could be able to deliver higher fractions of generated α -particles per targeted ^{225}Ac compared to the relative fractions of α -particles delivered by ^{225}Ac -labeled molecular carriers.

Keywords

actinium-225; bismuth-213; multivesicular liposomes; intraperitoneal alpha therapy; ovarian cancer micrometastases

*Corresponding author Stavroula Sofou, Ph.D., Polytechnic University, 6 MetroTech Center, Brooklyn, NY 11201, Phone: 718 260 3863, Fax: 718 260 3327, e-mail: ssofou@poly.edu.

[†] present address: Othmer-Jacobs Department of Chemical and Biological Engineering, Polytechnic University, 6 MetroTech Center, Brooklyn, NY 11201

This information is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Targeted α -particle emitters hold great promise as therapeutic agents for micrometastases (1). Alpha-particles are highly potent cytotoxic agents, potentially capable of tumor-cell kill without limiting morbidity. The increased effectiveness of α -particles is due to the amount of energy deposited per unit distance traveled (high LET), which is of the order of approximately 80 keV/ μ m. Cell survival studies have shown that α -particle-induced killing is independent of oxygenation state or cell-cycle during irradiation and that as few as 1–3 tracks across the nucleus may result in cell death (2–4). In addition, the 50- to 100- μ m range of α -particles is consistent with the dimensions of micrometastatic disseminated disease, allowing for localized irradiation of target cells with minimal normal-cell irradiation. Actinium-225 (^{225}Ac) is an α -particle emitter with increased cell killing efficacy (5–7), because each actinium-225 decay ($t_{1/2}=10$ d) generates three α -particle emitting daughters (^{221}Fr ($t_{1/2}=4.9$ min), ^{217}At ($t_{1/2}=32.3$ ms), ^{213}Bi ($t_{1/2}=45.59$ min)), and a total of 4 α -particles per decay. Thus, ^{225}Ac is an attractive candidate for α -particle therapy.

However, the optimal increase in cell killing efficacy of ^{225}Ac will occur only if all (or most of) α -emissions occur at the tumor site; otherwise, toxicity may be potentially increased. This is a fundamental difficulty if antibodies or other molecules with attached chelating ligands are to be used as the cell targeting vehicle, since the coordination bonds from the chelate to the ^{225}Ac atom will not be retained after decay of ^{225}Ac . This will leave the first daughter in the decay-chain free to distribute throughout the body where it and the resulting daughters will decay and increase toxicity. Thus, confinement of the intermediate radioactive daughters within the delivery carrier (during circulation) and at the tumor (after targeting) is desirable.

Liposomes have been previously considered for diagnostic and therapeutic delivery of radionuclides (8–10). We have previously investigated encapsulation of ^{225}Ac in liposomes as a means of retaining the α -particle-emitting daughters within liposomes during delivery (11). Because of their high kinetic energy, α -particle emissions will escape the liposomal phospholipid membrane to irradiate the targeted cells. Similarly, daughter atoms, during their recoil trajectory (80 to 90 nm), can also penetrate the phospholipid membranes. Because the newly produced daughter atoms are charged, after losing their recoil energy, they cannot diffuse freely across the hydrophobic compartment of the phospholipid bilayer. Consequently, if the end-point of a radioactive daughter's recoil trajectory is located within a liposome, the lipid membrane will inhibit free diffusion of the radioactive daughters, thereby retaining them at the site of liposome delivery. The probability of daughter retention is greater for larger liposomes assuming homogeneous distribution of the parent radionuclides within the liposomal aqueous core.

Our theoretical calculations (11) predicted negligible, <0.001 %, (last) daughter retention for 100 nm diameter liposomes and more than 50% retention (of the last daughter) for liposomes larger than 650 nm (in diameter). For giant liposomes with 1 μ m diameter, the maximum calculated retention of the last daughter does not exceed 65%. The measured last daughter retention (11 % decreasing to 7% after 10 days) of 650 nm diameter liposomes was not consistent with the theoretical prediction (50%), however, and this was shown to be caused, in part, due to binding of ^{225}Ac to the phospholipid membrane. Actinium-225 localization at the liposomal membrane increases daughter loss after nuclear recoil, compared to daughter loss from uniformly distributed ^{225}Ac atoms within the liposomal aqueous core. In this work we describe and characterize a construct designed to overcome this problem.

To increase daughter retention during circulation, ^{225}Ac was passively entrapped in MULTIVesicular Liposomes (MUVELs) (Fig 1). MUVELs are large liposomes (LLs) with entrapped small vesicles (SVs). Actinium-225 was contained in the small vesicles only. This

strategy provides: (1) confinement of entrapped ^{225}Ac within the aqueous compartment of the large liposomes (LLs) where the small vesicles are contained, and, thus, away from the membrane of large liposomes, and (2) decreased radionuclide partition to the external membrane (large liposome membrane), due to increased internal membrane owed to the encapsulated small vesicles. Retention of ^{225}Ac and its last radioactive daughter ^{213}Bi by MUVEL was evaluated over time. In these studies, MUVELs were PEGylated to assure long blood circulation times (12). MUVELs were then labeled with the monoclonal anti-HER2/neu antibody Trastuzumab (immunolabeled MUVELs), and were evaluated *in vitro* for targeted delivery of ^{225}Ac to ovarian carcinoma cells (SKOV3-NMP2).

Experimental Procedures (Materials and Methods)

Reagents

The lipids 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC), 1,2-Diheneicosanoyl-*sn*-Glycero-3-Phosphocholine (21:0PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (ammonium salt) (PEG-labeled lipid), L- α -phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (egg) (rhodamine-PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol) 2000] (ammonium salt) (maleimide-PE) (purity >99%) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, phosphate buffered saline (PBS), fluorexon (calcein), Sephadex G-50, diethylenetriaminepentaacetic acid (DTPA), ascorbic acid, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). p-isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was purchased from Macrocyclics (Dallas, TX). Traut's reagent (2-Iminoethiolane-HCl) was purchased from Pierce (Rockford, IL). Actinium-225 nitrate was purchased from the Department of Energy, Oak Ridge National Laboratories (Oak Ridge, TN).

Liposome preparation

The protocol to prepare MUVELs involves passive entrapment of small vesicles (SVs) into large liposomes (LLs). The phospholipid-cholesterol combinations chosen for the encapsulated small vesicles (SVs) and the large liposomes (LLs) were those that resulted in: (1) the lowest release of contents by SVs after annealing at 40°C (40°C annealing is required for the hydration of the membranes of the LLs that contain encapsulated SVs), (2) the greatest passive entrapment efficacy by the LLs, and (3) the minimum fraction of fusion between lipids of SVs and LLs.

To make small vesicles, mixtures of phosphatidyl choline (21:0PC), cholesterol (1:1 molar ratio), and PEG-labeled lipids (5.3 mole % of total lipid) in CHCl_3 were dried in a rotary evaporator. For ^{225}Ac passive entrapment, the lipids (30 mM final concentration) were resuspended in PBS (1 mM EDTA, pH = 7.4) containing the chelated actinium complex (^{225}Ac -DOTA, 3.7–37.0 MBq, depending on radionuclide availability) and DTPA (1 mM). DTPA was used to potentially chelate 'free' ^{225}Ac and ^{213}Bi . The protocol for ^{225}Ac -DOTA chelation is published elsewhere (5). The lipid suspension was then annealed to 55°C for 2 hours. Small vesicles were prepared by brief sonication (1–2 min) of the lipid suspension in a bath sonicator (Branson, Danbury, CT) at 75–80°C until appearance of a clear solution. The vesicle suspension was then incubated at room temperature for 15 minutes with 0.050 ml of externally added 10 mM DTPA to complex any free metals before purification during size exclusion chromatography (SEC) in a Sephadex G-50 (Aldrich, St. Louis, MO) packed 1×10 cm column, eluted with sucrose isotonic PBS.

Then to prepare MUVELs, small vesicles were passively encapsulated into large liposomes (LLs). For LLs, mixtures of phosphatidyl choline (DMPC), cholesterol (2:1 molar ratio),

maleimide-PEG- labeled lipids (1 mole % of total lipid) and PEG-labeled lipids (4.3 mole % of total lipid) in CHCl_3 were dried in a rotary evaporator. For passive entrapment of small vesicles, the dry lipids (60 mM final lipid concentration) were resuspended in the isosmolar sucrose-PBS suspension containing small vesicles (described above). The lipid suspension was then annealed to 40°C for 2 hours. MUVELs were prepared by the extrusion method. The lipid suspension was taken through twenty-one cycles of extrusion (LiposoFast, Avestin, Ontario, Canada) through two stacked polycarbonate filters (800 nm filter pore diameter), and then diluted in sucrose-free isosmotic PBS solution. Unentrapped small vesicles were removed by centrifugation, and the majority of MUVELs (90% as calculated by the fluorescent intensity of rhodamine-labeled lipids in parallel measurements) were collected in the pellet.

Plain large liposomes (LLs) were prepared as above, without encapsulating small vesicles.

In all preparations, ascorbic acid (8 mmol/l) was coentrapped to minimize lipid oxidation due to gamma- (13) and beta-radiation (14), and possibly due to alpha-emissions (15,16).

Liposome size distribution determination

For dynamic light scattering (DLS), an N4 Plus autocorrelator (Beckman-Coulter) was used, equipped with a 632.8 nm He-Ne laser light source. The measurement protocol is published elsewhere (11).

Cryo-TEM

MUVELs were imaged using a FEI Tecnai 20 cryo-TEM. Measurements were performed by the staff of the Analytical Imaging Facility at the Albert Einstein College of Medicine, Yeshiva University. Samples were frozen and thin frozen sections were imaged without staining.

Retention of entrapped contents by liposomes

The protocol to measure the retention of ^{225}Ac and its last radioactive daughter ^{213}Bi by liposomes is described, in detail, elsewhere (11). Briefly, the γ -emissions (radioactivity increase over time and equilibrium values) of ^{213}Bi were measured in liposome fractions, which were separated, at different times, from the parent liposome population and the free radionuclides by SEC (Sephadex G-50). The samples were counted using a Cobra γ -counter (Packard Instrument Co., Inc.). For monitoring ^{213}Bi the energy window 360 – 480 keV was used, and for ^{225}Ac the window 150–600 keV, which includes the chief ^{221}Fr and ^{213}Bi photopeaks. Repeated measurements were performed on three different preparations of each liposome structure.

Liposome immunolabeling

The protocol to immunolabel liposomes is described elsewhere (17). Briefly, Trastuzumab purified from Herceptin® (Genentech, South San Francisco, CA), or Rituximab® (used as isotype-matched control mAb to Trastuzumab in the flow cytometry studies), was activated with Traut's reagent, and was reacted with maleimide-lipids contained in liposomes (1 mole % of total lipid). In purified immunoliposome suspensions (after SEC in a 4B Sepharose, 1×10 cm), a protein assay was used to quantify the concentration of antibodies, and lipid concentration was determined by the fluorescence intensity of rhodamine-lipids contained in liposomes (0.5–1 mole% of total lipid). Using the measured values of conjugated antibodies-per-lipid, the average number of antibodies per liposome was calculated (17) using for input values the mean size of liposomes (as measured by DLS), and the head group surface area per lipid ($70\text{\AA}^2$)(18).

Trastuzumab was also directly radiolabeled with ^{225}Ac . This preparation protocol is published elsewhere (5,19). Conventional labeling of a DOTA-IgG construct does not yield stable product

in high yield under any conditions (19,20) which lead to the development of the 2-step labeling method. Briefly, the antibody was radiolabeled in 2-steps: the first step entails ^{225}Ac + DOTA-NCS, which proceeded to >95% completion in 30 min.; the second step involves the conjugation of the reactive isothiocyanate moiety on the ^{225}Ac -DOTA-NCS product with lysines on the Trastuzumab. This synthetic path was used as the ^{225}Ac + DOTA chelation only goes to completion at elevated temperatures, 55–60°C, a temperature that will denature the IgG.

Immunoreactivity—Cells were washed twice with ice-cold PBS and blocked by incubation on ice with 2% BSA. Then, ^{225}Ac -labeled antibody (2.6 ng) in 1% HSA, small vesicles and liposomes (LLs and MUVELs) at 1.0–1.9 micrograms and 100–190 micrograms total lipid, were added to 10^7 cells, and incubated on ice for 30 min. Cells were then washed twice with ice-cold PBS and centrifuged. Twenty-four hours later, supernatants and pellets were counted by scintigraphy (Beckman-Coulter, Fullerton, CA).

Cell line

The metastatic ovarian carcinoma cell line, SKOV3-NMP2 was derived from serial passage of the parental SKOV3 cell line in nude mice (21), and was used. Stock T-flask cultures (20) were propagated at 37°C, in 5% CO_2 in RPMI 1640 media supplemented with 10% fetal calf serum (Sigma-Aldrich), 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cell concentration was determined by counting trypsinized cells with a hemocytometer.

Cell Binding and internalization of liposomes and radiolabeled antibody

Harvested SKOV3-NMP2 cells were washed twice with ice-cold media (RPMI 1640/10% FBS/2% BSA) and then resuspended in ice-cold media at a density of 10^6 cells/ml. Radiolabeled liposomes (0.1 – 1.8 μM lipid, final concentration) or antibody (25 ng) were added to 3.5 ml of cell suspension and two 200 μl samples were immediately taken and processed as described below. The cells were then placed in a humidified, 37°C incubator with 5% CO_2 , where they were periodically swirled and sampled at 0.5, 1, 2, 4 and 24 hours. The cells were washed three times with 2 ml of ice-cold PBS, and then 1 ml of an acidic stripping buffer (50 mM glycine, 150 mM NaCl, pH=2.8) was added for 10 minutes at room temperature to eliminate the charge-specific binding of membrane bound conjugates and to remove the surface bound immunoliposomes or antibodies. After centrifugation, supernatants and pellets were allowed to reach equilibrium before counting (20 h).

Flow cytometry

Liposomal membranes were labeled with the fluorescent lipid rhodamine-PE (excitation: 550 nm; emission: 590 nm). Harvested cells (ovarian cancer SKOV3-NMP2 cells and fibroblast AL67 cells) were washed three times with ice-cold buffer (PBS/0.5% BSA/0.02% NaN_3) and then resuspended at a density of 5.6×10^6 cells/ml. 10^6 cells were incubated on ice with liposomes for 25 minutes (3.3 mM final lipid concentration), then washed three times and finally resuspended at a density of 2.5×10^6 cells/ml. To determine the extend of specific binding of immunoliposomes to the HER2/neu antigen receptor, cells were also preincubated with Trastuzumab at 5 μg of antibody per one million cells for 25 minutes on ice. Cells were then washed twice with ice-cold buffer and incubated with liposomes as above. Fluorescence counting of cell suspensions (50,000 events) was measured using a Beckman-Coulter Cytonics FC500 flow cytometer (Fullerton, CA), and analyzed with the software FlowJo (Tree Star, Inc., Ashland, OR).

Results

Retention of ^{225}Ac by Liposomal Structures

In multivesicular liposomes (MUVELs), ^{225}Ac was encapsulated only into the small vesicles (SVs). For 30 days (Figure 2, upper part), more than 95% of the encapsulated ^{225}Ac activity was retained by MUVELs (black triangles) and SVs (black circles). In large liposomes (LLs), only 70% of ^{225}Ac was retained stably over time (black squares).

The retention of ^{225}Ac by liposomes depends on the permeability of the liposomal membrane to the ^{225}Ac -DOTA complex and is not dependent on the radioactive character of ^{225}Ac which after its recoil may eject the next daughter out of the physical boundaries of liposomes (as is the case for ^{213}Bi). The results on ^{225}Ac retention are identical both for MUVELs and for the small vesicles (SVs) (Figure 2). In both of these cases ^{225}Ac is encapsulated into the same rigid membranes composed of diheptacosanoyl phosphocholine (21:0 PC, $T_g=72^\circ\text{C}$) and cholesterol. Small vesicles (SVs) are comprised of this lipid composition. In MUVELs, the encapsulated small vesicles (SVs) that contain ^{225}Ac are also comprised of exactly this lipid composition. On the other hand, Large Liposomes (LLs) are comprised of more fluid lipids (dimyristoyl phosphocholine, 16:0 $T_g=23^\circ\text{C}$; and cholesterol) that appear to have more permeable membranes to ^{225}Ac -DOTA complexes.

Retention of ^{213}Bi by Liposomal Structures

MUVELs (white triangles) and LLs (white squares) retained over a period of 30 days (Figure 2, lower part) $17 \pm 3\%$ and $15 \pm 7\%$ of ^{213}Bi , respectively (approximately one-third of the theoretical maximum)(11). Both MUVELs and LLs exhibited almost identical ^{213}Bi retention profiles per encapsulated ^{225}Ac nucleus suggesting that the particular composition of the (external) liposomal membrane does not promote radionuclide localization as opposed to the lipid composition chosen in our first studies (11). However, as shown above (Figure 2, upper part), LLs failed to adequately retain the parent ^{225}Ac . Therefore, MUVELs increase the fraction of overall delivered daughters. For example on day 20, the retention of ^{213}Bi by MUVELs is 17% and by LLs is 15% of the total ^{213}Bi per encapsulated ^{225}Ac . The retention of ^{225}Ac by MUVELs is 94% and by LLs is 73% of the initially encapsulated radioactivity. Therefore, in terms of the initially encapsulated ^{225}Ac radioactivity: $0.17 \times 0.94 = 16\%$ and $0.15 \times 0.73 = 11\%$ of ^{213}Bi is delivered at the site of MUVELs and LLs, respectively.

Also, consistent with our previous theoretical predictions, is the insignificant retention of ^{213}Bi (white circles) by small vesicles with sizes comparable to the recoil range of the α -emitting daughters (22).

Liposome and Antibody Radiolabeling and Quality Control

More than 95% of ^{225}Ac was chelated to DOTA. The maximum encapsulation efficiency of ^{225}Ac by the liposomal structures using passive entrapment did not exceed 10% of the total initial activity.

The efficiency of radioconjugation to antibody was low with radiochemical yield 3% (of the total activity applied) and resulted in radiolabeled antibodies with low specific activity, 1.406 KBq/microgram. The radiosynthesis of the ^{225}Ac -DOTA-IgG constructs has been described (19) and while the products were radiochemically pure and biologically reactive the yields were low (<10%). The antibody was radiolabeled in 2-steps and the specifications and yields of the ^{225}Ac -DOTA-Trastuzumab construct were similar (20,23) to the other constructs tested (19). The low yield in the second step results from the competing hydrolysis reaction of the isothiocyanate moiety. The mass amounts of DOTA and IgG as well as the activity amount of ^{225}Ac were optimized (19) and adding more or less of any of these components will not

significantly improve the overall yield. The reported specific activity 1.406 kBq/microgram (0.038 Ci/g) was on the same order as the specific activities used clinically with the ^{225}Ac -DOTA-HuM195 therapeutic drug (24) and these 'small' doses have demonstrated anti-leukemic effects.

The conjugation reaction resulted in 937 ± 110 antibodies per liposome. Leakage of entrapped radioactive or fluorescent (calcein) contents due to conjugation, was not detected (data not shown). The measured average large liposome size for MUVELs was 758 ± 287 nm.

The Her2/neu cell-surface density of $1-3 \times 10^5$ per cell was used (25), and approximately one Trastuzumab molecule per 100–300 cell receptors were mixed. The immunoreactivity of radiolabeled Trastuzumab was 65.74 ± 7.51 %. For liposomes, two ratios were tested: (a) one hundred liposomes per cell (or one liposome-conjugated antibody per 1–3 receptors), and (b) one liposome per cell (or one liposome-conjugated antibody per 100–300 receptors). The antigen binding activity of liposomes was similar for both ratios tested: 4.40 ± 0.89 % of immunolabeled MUVELs, and 1.83 ± 0.52 % of immunolabeled LLs were bound to cells at both lipid-to-cell ratios. Small vesicles (SVs), that were not immunolabeled, bound non-specifically at a low extent (0.75 ± 0.02 %).

Cell Binding and Internalization

After 4 hours of incubation, the relative total cell-associated radioactivities (Figure 3, closed symbols), compared to $t=0$, were a 2-, 7- and 135-fold higher for LLs (closed squares), MUVELs (closed circles), and the radiolabeled antibody (closed triangles), respectively. Both types of immunoliposomes (MUVELs and LLs) internalized to a greater extent than the antibody: 83% of the activity of bound immunolabeled MUVELs, and 70% of the activity of bound immunolabeled LLs were internalized (4 h incubation) as opposed to only 23% of bound antibody. Also, the fraction of total cell-associated activity (^{225}Ac) delivered by immunolabeled MUVELs (2.5 ± 0.5 %) was 3.8 times greater than the fraction of total associated radioactivity delivered by immunolabeled LLs (0.7 ± 0.1 %), probably due to the less stable retention of the parent nuclide by LLs (Figure 2, upper part). For the same incubation time, the fraction of total cell-associated activity for non-immunolabeled MUVELs, LLs and SVs was 0.47%, 0.61% and 0.16% of the total added radioactivity, respectively (data not shown). Cell uptake kinetics was significantly slower for all liposome structures compared to the radiolabeled antibody.

Using flow cytometry, significant shift in fluorescence counts (one log unit) was detected only when anti-HER2/neu-immunoliposomes were incubated with SKOV3-NMP2 cells with unblocked HER2/neu receptors (Figure 4A, thick line). Neither specific nor non-specific binding of liposomes to AL67 (fibroblast cells not expressing the HER2/neu receptor) was detected. The specificity of binding of Trastuzumab-conjugated liposomes to SKOV3-NMP2 cells was also compared to the binding of liposomes conjugated to Rituximab[®], an anti-CD20 antibody that was used as the isotype-matched control to Trastuzumab. Trastuzumab-labeled liposomes resulted in a shift of fluorescence counts of SKOV3-NMP2 cells that was more than one log unit greater than the shift measured for Rituximab[®]-labeled liposomes. (flow data shown in supplemental data section).

DISCUSSION

Multivesicular liposomes (MUVELs) result in minimal leakage of ^{225}Ac and in significant retention of the α -emitting daughters. MUVELs with encapsulated ^{225}Ac deliver 2.8 out of 4 α -particles emitted per ^{225}Ac decay. This ratio is calculated based on the assumption that the last two radioactive daughters have the same probability 'x' to be retained within liposomes after decay. Assuming probability one for the first daughter ^{221}Fr , since ^{225}Ac should be

confined close to the core of the large liposomes with radius larger than the daughter's recoil distance, for the four decays (^{225}Ac to ^{221}Fr , ^{221}Fr to ^{217}At , ^{217}At to ^{213}Bi , and ^{213}Bi to stable ^{209}Pb) the probability of daughter retention (and thus targeted delivery of the subsequently emitted alphas) is x^2 . From Figure 2, $1 * x^2$ is approximately 0.18, x should be equal to 0.42, and the total alphas retained should equal to $1 + 1 + 0.42 * 2 = 2.8$.

We have previously calculated that for liposomes as large as 750 nm in diameter (similar to the sizes of MUVEL's and LL's) not more than 55% of generated ^{213}Bi from the total ^{213}Bi generated by the encapsulated ^{225}Ac is retained by liposomes, and can therefore be expected to decay at the cite of the liposome carriers (11). These calculations were based on the average recoil trajectory of each daughter (80–90 nm) that is comparable to the size of the liposomal carriers. For this geometry, the probability that a generated daughter would be retained within a liposome, decreases as the previous radioactive daughter's location approaches the liposome membrane at the time point of the radionuclide decay. In addition, for every subsequent radioactive daughter the probability of remaining within the physical limits of the liposomal carrier is decreasing compared to the probability corresponding to the previous radioactive daughter. MUVELs exhibit retention of ^{213}Bi that is 17–18% of the total ^{213}Bi generated by the encapsulated ^{225}Ac . In other words, these values correspond to 31–33 % of the theoretical maximum retention for this size of delivery carriers.

In vitro, immunolabeled MUVELs demonstrated greater specific binding to human ovarian carcinoma cells (SKOV3-NMP2) compared to non-targeted liposomes, but showed lower 'immunoreactivity' than the radiolabeled antibody. This result could be due to the relatively large size of liposomes that may interfere with the extracellular matrix by obstructing free diffusion towards the cell surface. The targeting antibodies were conjugated on the free-ends of surface-grafted PEG-chains of the same length (26), to exclude steric hindrance to targeting from neighboring grafted PEG-chains. Also, cell uptake kinetics was slower for immunoliposomes compared to free antibody that could be justified by the different diffusivities of each structure studied. Measured diffusion coefficients of free IgG's are of the order of $10^{-7} \text{ cm}^2/\text{sec}$ in water at 20°C (27). At the same conditions, the calculated values for liposomes (using the Stokes-Einstein equation for spherical objects) are two orders of magnitude lower ($10^{-9} \text{ cm}^2/\text{sec}$).

In ^{225}Ac -radioimmunotherapy, renal toxicity can become a major dose constraint due to localization of escaped ^{213}Bi in the kidneys (28,29). MUVELs are proposed for retaining the radioactive daughters at the site of the carrier for the interval between administration of the carriers and before the carriers are internalized by the targeted cancer cells. Such radioimmunoliposomes would reduce the fraction of escaped radioactive daughters and could potentially be used for the therapy of disseminated micrometastatic tumors in the peritoneal cavity using direct intraperitoneal administration. This approach could combine the following beneficial features: (a) targeting efficacy and specificity due to surface-conjugated tumor-specific antibodies (17,30); (b) long retention times in the peritoneal cavity (31,32), due to large liposome sizes; (c) direct tumor access (20,33,34) combined with a radiation dose range tailored to micrometastases that do not have developed vasculature (35) and cannot be differently accessed; and (d) minimal irradiation of the surrounding healthy tissues due to the α -particles' comparatively short range.

The stable retention of ^{225}Ac by MUVELs will probably be challenged in the presence of serum proteins *in vivo*, since proteins are known to interact with the lipid membrane of these carriers in a variety of ways (36). Preliminary *in vivo* biodistributions of ^{225}Ac -loaded immunolabeled-liposomes (MUVELs and LLs) that were administered intraperitoneally in BALB/c nude mice bearing intraperitoneally disseminated SKOV3-NMP2 tumors resembling micrometastatic disease (37), suggest that *in vivo* MUVELs retain ^{225}Ac to a larger extent than LLs, and

therefore deliver higher activities of ^{213}Bi to the tumor sites. In addition, both types of immunoliposome compositions exhibit significant hepatic and splenic uptake that is characteristic of this size of drug carriers and could determine the maximum tolerated dose. The short range of alpha-particles emitted at the sites of normal organs could, however, result in relatively low toxicities at these organs due to the short range of alpha-particles.

Finally, immunolabeled MUVELs may be particularly useful in delivering lethal radiation doses to cancer cells with low expression levels of molecular targets (38). Actinium-225 labeled antibodies have generally low specific activity (1.406 MBq/mg in this study) that corresponds to one conjugated ^{225}Ac atom per 2,300 antibodies. For MUVELs, two passive entrapment steps are required (each with a maximum of 10% encapsulation efficiency). Thus, for 370 MBq (10mCi) ^{225}Ac initial activity, and 1% actinium overall entrapment efficacy, we can encapsulate one actinium nuclide per MUVEL and two actinium nuclides in every 8 MUVELs (for 4×10^{12} liposomes with a mean diameter of 750nm). Our current work is focused on increasing the encapsulated ^{225}Ac activity within small vesicles using active (ionophore-driven) loading (39). Additional structural optimization of MUVELs is also required to further increase ^{213}Bi retention at the liposome sites.

Intraperitoneal micrometastatic disease constitutes a treatment challenge, and is common among patients with advanced gynecological and gastrointestinal cancers. In this work, the ovarian carcinoma cells SKOV3-NMP2 were selected to prove, *in vitro*, the feasibility of targeted delivery of the α -particle nanogenerator ^{225}Ac and its radioactive daughters using immunolabeled MUVELs that can potentially be used against disseminated intraperitoneal micrometastases following locoregional administration. Our findings show that immunolabeled MUVELs retain a third of the theoretical maximum of the radioactive daughters, and the cell bound structures become considerably more internalized by ovarian cancer cells than the radiolabeled antibody. These results suggest that the effective numbers of α -particles emitted at the target per delivered ^{225}Ac using MUVELs could be significant, thus providing a promising therapeutic modality for disseminated micrometastatic disease. Additional optimization of MUVELs is necessary to increase the encapsulated radioactivity of ^{225}Ac in order to enable the evaluation of these liposomes' potential for therapeutic use. Additional increase in daughter retention could be achieved by further increasing the size of the delivery carrier (to 1 micron diameter), but given the limitations of stable intact liposomes of such large diameters, probably different materials could be evaluated to form the outer large shell encapsulating Small Vesicles (SVs) with entrapped ^{225}Ac .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Work supported by the USAMRMC Concept Award DAMD170010657, USAMRMC IDEA Award DAMD170310755, NIH R01 CA55349, the Experimental Therapeutics Center, and the Goodwin Commonwealth Foundation for Cancer Research. D.A.S. is a Doris Duke Distinguished Science Professor. S.S. is the recipient of Dr. Frederick E.G. Valergakis Graduate Research Grant of the Hellenic University Club of New York.

References

1. Couturier O, Supiot O, Degraef-Mouglin M, Faivre-Chauvet A, Carlier T, Chatal JF, Cherel M. Cancer radioimmunotherapy with alpha-emitting nuclides. *Eur J Nucl Med Imaging* 2005;32:601–614.
2. Humm JL. A microdosimetric model of astatine-211 labeled antibodies for radioimmunotherapy. *Int J Radiat Oncol Biol Phys* 1987;13:1767–1773. [PubMed: 3667382]

3. Humm JL, Chin LM. A model of cell inactivation by alpha-particle internal emitters. *Radiat Res* 1993;134:143–150. [PubMed: 8488249]
4. Macklis RM, Kinsey BM, Kassis AI, Ferrara JL, Archer RW, Hines JJ, Coleman CN, Adelstein SJ, Burakoff SJ. Radioimmunotherapy with alpha-particle-emitting immunoconjugates. *Science* 1988;240:1024–1026. [PubMed: 2897133]
5. McDevitt MR, Ma D, Lai LT, Simon J, Borchardt P, Frank RK, Wu K, Pellegrini V, Curcio MJ, Miederer M, Bander NH, Scheinberg DA. Tumor therapy with targeted atomic nanogenerators. *Science* 2001;294:1537–1540. [PubMed: 11711678]
6. Kennel SJ, Brechbiel MW, Milenic DE, Schlom J, Mirzadeh S. Actinium-225 conjugates of MA6 CC49 and humanized delta CH2CC49. *Cancer Biother Radiopharm* 2002;17:219–231. [PubMed: 12030116]
7. Kennel SJ, Chappell LL, Dadachova K, Brechbiel MW, Lankford TK, Davis IA, Stabin M, Mirzadeh S. Evaluation of Ac-225 for vascular targeted radioimmunotherapy of lung tumors. *Cancer Biother Biopharm* 2000;15:235–244.
8. Emfietzoglou D, Kostarelos K, Papakostas A, Yang WH, Ballangrud A, Song H, Sgouros G. Liposome-mediated radiotherapeutics within avascular tumor spheroids: comparative dosimetry study for various radionuclides, liposome systems, and a targeting antibody. *J Nucl Med* 2005;46:89–97. [PubMed: 15632038]
9. Kostarelos K, Emfietzoglou D, Papakostas A, WH Y, Ballangrud AM, Sgouros G. Engineering lipid vesicles of enhanced intratumoral transport capabilities: correlating liposome characteristics with penetration into human prostate tumor spheroids. *J Liposome Res* 2005;15:15–27. [PubMed: 16194925]
10. Zielhuis SW, Nijssen JFW, Seppenwoodle JH, Zonnenberg BA, Bakker CJG, Hennink WE, van Rijk PP, van het Schip AD. Lanthanide bearing microparticulate systems for multi-modality imaging and targeted therapy of cancer. *Curr Med Chem- AntiCancer Agents* 2005;5:303–313. [PubMed: 15992356]
11. Sofou S, Thomas JL, Lin HY, McDevitt MR, Scheinberg DA, Sgouros G. Engineered liposomes for potential α -particle therapy of metastatic cancer. *J Nucl Med* 2004;45:253–260. [PubMed: 14960644]
12. Allen C, Dos Santos N, Gallagher R, Chiu GNC, Shu Y, Li WM, Johnstone SA, Janoff AS, Mayer LD, Webb MS, Bally MB. Controlling the physical behavior and biological performance of liposome formulations through use of surface grafted poly(ethylene glycol). *Bioscience Rep* 2002;22:225–250.
13. Stensrud G, Redford K, Smistad G, Karlsen J. Effects of gamma irradiation on solid and lyophilised phospholipids. *Rad Phys Chem* 1999;56:611–622.
14. Chakrabarti MC, Le N, Paik CH, De Graff WG, Carrasquillo JA. Prevention of radiolysis of monoclonal antibody during labeling. *J Nucl Med* 1996;37:1384–1388. [PubMed: 8708780]
15. McDevitt MR, Finn RD, Sgouros G, Ma D, Scheinberg DA. An Ac-225/Bi-213 generator system for therapeutic clinical applications: construction and operation. *Appl Radiat Isot* 1999;50:895–904. [PubMed: 10214708]
16. Nikula TK, McDevitt MR, Finn RD, Wu C, Kozak RW, Garmestani K, Brechbiel MW, Curcio MJ, Pippin CG, Tiffany-Jones L, Geerlings SMW, Apostolidis C, Molinet R, Geerlings JMW, Gansow OA, Scheinberg DA. Alpha-emitting bismuth cyclohexylbenzyl DTPA constructs of recombinant humanized anti-CD33 antibodies: pharmacokinetics, bioactivity, toxicity and chemistry. *J Nucl Med* 1999;40:166–176. [PubMed: 9935073]
17. Kirpotin DB, Park JW, Hong SK, Zalipsky S, Li WL, Carter P, Benz CC, Papahadjopoulos D. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro*. *Biochemistry* 1997;36:66–75. [PubMed: 8993319]
18. Lasic, DD. *Liposomes from Physics to Applications*. Elsevier; Amsterdam: 1993.
19. McDevitt MR, Ma D, Simon J, Frank RK, Kiefer GE, Scheinberg DA. Design and synthesis of actinium-225 radioimmunopharmaceuticals. *Appl Radiat Isot* 2002;57:841–847. [PubMed: 12406626]
20. Borchardt P, Yuan R, Miederer M, McDevitt M, Scheinberg D. Targeted actinium-225 in vivo generators for therapy of ovarian cancer. *Cancer Res* 2003;63:5084. [PubMed: 12941838]
21. Mujoo K, Maneval DC, Anderson SC, Gutterman JU. Adenoviral-mediated p53 tumor suppressor gene therapy of human ovarian carcinoma. *Oncogene* 1996;12:1617–1623. [PubMed: 8622881]

22. Ziegler, J. Stopping and range of ions in matter (SRIM96). Yorktown, New York: IBM; 1996.
23. Ballangrud Å, Yang WH, Palm S, Enmon R, Borchardt P, Pellegrini V, McDevitt M, Scheinberg D, Sgouros G. Alpha-particle Emitting Atomic Generator (Actinium-225)-Labeled Trastuzumab (Herceptin) Targeting of Breast Cancer Spheroids: Efficacy versus HER2/neu Expression. *Clin Cancer Res* 2004;10:4489–4497. [PubMed: 15240541]
24. Jurcic J, McDevitt M, Divgi C, Pandit-Taskar N, Finn R, Sgouros G, Chanel S, Larson S, Scheinberg D. Alpha-Particle Immunotherapy for Acute Myeloid Leukemia (AML) with Bismuth-213 and Actinium-225. *Cancer Biother Radiopharm* 2006;21:s396.
25. Dean GS, Pusztai L, Xu FJ, O'Briant K, DeSombre K, Conaway M, Boyer CM, Mendelsohn J, Bast RCJ. Cell surface density of p185c-erbB-2 determines susceptibility to anti-p185c-erbB-2-Ricin A chain (RTA) immunotoxin therapy alone and in combination with anti-p170EGFR-RTA in ovarian cancer cells. *Clin Cancer Res* 1998;4:2545–2550. [PubMed: 9796989]
26. Maruyama K, Takizawa T, Yuda T, Kennel SJ, Huang L, Iwatsuru M. Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies. *Biochim Biophys Acta* 1995;1234:74–80. [PubMed: 7880861]
27. Saltzman WM, Radomsky ML, Whaley KJ, Cone RA. Antibody diffusion in human cervical mucus. *Biophys J* 1994;66:508–515. [PubMed: 8161703]
28. Jaggi J, Seshan SV, McDevitt MR, LaPerle K, Sgouros G, Scheinberg DA. Renal tubulointerstitial changes after internal irradiation with alpha-particle-emitting actinium daughters. *J Am Soc Nephrol* 2005;16:2677–2689. [PubMed: 15987754]
29. Jaggi J, Kappel BJ, McDevitt MR, Sgouros G, Flombaum CD, Cabassa C, Scheinberg DA. Efforts to control the errant products of a targeted in vivo generator. *Cancer Res* 2005;65:4888–4895. [PubMed: 15930310]
30. Sapra P, Allen TM. Ligand-targeted liposomal anticancer drugs. *Prog Lipid Res* 2003;42:439–462. [PubMed: 12814645]
31. Sadzuka Y, Hirota S, Sonobe T. Intraperitoneal administration of doxorubicin encapsulating liposomes. *Toxicology letters* 2000;116:51–59. [PubMed: 10906422]
32. Verschraegen C, Kumagai S, Davidson R, Feig B, Mansfield P, Lee S, Maclean D, Hu W, Khokhar A, Siddik Z. Phase I clinical and pharmacological study of intraperitoneal cis-bis-neodecanoato (trans- R, R-1, 2-diaminocyclohexane)-platinum II entrapped in multilamellar liposome vesicles. *J Cancer Res Clin Oncol* 2003;129:549–555. [PubMed: 14513369]
33. Buijs WCAM, Tibben JG, Boerman OC, Molthoff CFM, Massuger LFAG, Koenders EB, Schijf CPT, Siegel JA, Corstens FHM. Dosimetric analysis of chimeric monoclonal antibody cMOv18 IgG in ovarian carcinoma patients after intraperitoneal and intravenous administration. *Eur J Nucl Med* 1998;25:1552–1561. [PubMed: 9799353]
34. Markman M. Role of intraperitoneal chemotherapy in the front-line setting. *J Clin Oncol* 2003;21:145s–148s. [PubMed: 12743130]
35. Li CY, Shan S, Huang Q, Braun RD, Lanzen J, Hu K, Lin P, Dewhirst MW. Initial Stages of Tumor Cell-Induced Angiogenesis: Evaluation Via Skin Window Chambers in Rodent Models. *J Natl Cancer Inst* 2000;92:143–147. [PubMed: 10639516]
36. Lasic, DD.; Martin, F. CRC Press; Boca Raton, Fla: 1995. p. 289
37. Sofou S, Enmon RM, McDevitt MR, Jaggi J, Thomas JL, Scheinberg DA, Sgouros G. Multivesicular radioimmunoliposomes with encapsulated Actinium-225 for targeted alpha-particle therapy of intraperitoneal micrometastatic cancer. *Cancer Biother Radiopharm* 2004;19:s518–519.
38. McQuarrie SA, Mercer JR, Syme A, Suresh MR, Miller GG. Preliminary results of nanopharmaceuticals used in the radiotherapy of ovarian cancer. *J Pharmaceut Sci* 2004;7:29–34.
39. Chang MY, Sgouros G, Sofou S. Enhanced liposomal encapsulation of actinium-225: High loading activities and stable retention for potential therapeutic use. *Cancer Biother Radiopharm* 2006;21:s394.

Figure 1.

Cryo-TEM image of multivesicular liposomes (horizontal edge is approximately 400 nm), and schematic representation of MUVELs and their components.

Figure 2. Fraction of ^{225}Ac retention (upper left, closed symbols) and ^{213}Bi retention per encapsulated ^{225}Ac (lower right, open symbols) by multivesicular liposomes (MUVELs) (triangles), large liposomes (LLs) (squares), and small vesicles (SVs) (circles) during 30 d. The error bars correspond to standard deviations of measurements performed on three different preparations of each liposome structure.

Figure 3.

Cell binding and internalization of immunolabeled lipid-structures with encapsulated ^{225}Ac , and ^{225}Ac -conjugated antibody. Closed symbols: fraction of the total initial-activity that became cell-associated. Open symbols: fraction of cell-internalized activity. Triangles: radiolabeled antibody; Circles: immunolabeled MUVELs; Squares: immunolabeled LLs. The error bars correspond to standard deviations of measurements performed in duplicates.

Figure 4. Binding of rhodamine-lipid-containing Trastuzumab-labeled liposomes to SKOV3-NMP2 (A) and AL67 cells (B). Shaded grey: cells; Thin line: cell fluorescence due to immunoliposomes bound to cells that have been pre-incubated with Trastuzumab; Thick line: cell fluorescence due to immunoliposomes bound to cells with unblocked receptors.