Sterically stabilized liposomes as a carrier for α-emitting radium and actinium radionuclides

Abbreviated title: Liposomal carrier for α-emitting Ra and Ac

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Abstract

The α-particle emitting radionuclides ^{223}Ra ($t_{½}$ = 11. 4 d), ^{224}Ra ($t_{½}$ = 3.6 d) and ^{225}Ac($t_{½}$ = 10.0 d) may have a broad application in targeted radiotherapy provided they could be linked to vehicles with tumor affinity. The potential usefulness of liposomes as carriers was studied in the present work. Radium and actinium radionuclides could be loaded in good yields into sterically stabilized lipsomes. Subsequent coating of the lipsomes with a folate-F(ab')$_2$ construct yielded a product with affinity towards tumor cells expressing folate receptors. Radionuclide loaded liposomes showed excellent stability in serum in vitro.

1. Introduction

The strong cytotoxicity of high linear energy transfer (LET) α-particle radiation [1,2], with a range in tissue corresponds to a few cell diameters, may be exploited in targeted radiotherapy of cancer [3-5] to minimize the dose to normal tissues adjacent to the malignant lesions. For targeted α-particle radionuclide therapy to be effective to combat solid tumors, the radioactivity should be distributed as evenly as possible within the tumor.

There are, however, physiological barriers slowing down the uptake and distribution of a high molecular weight carrier molecules in solid tumors [6]. In a few diseases a rapid targeting is currently possible making clinical studies with the short-lived nuclides ^{211}At ($t_{1/2}$ = 7.2 h) [7] and ^{213}Bi ($t_{1/2}$ = 45.6 min) [8] feasible, but for many cancer forms these nuclides may have to short half-lives. For instance, tumor uptake with systemically administered IgG molecules often reaches a maximum after more than 1 day rendering the short-lived nuclides therapeutically unsuitable in such systems. The use of alpha-emitters having a significant longer half-life than those previously used clinically may thus be beneficial. Firstly, it may improve the ratio between radiation dose to tumor and normal tissues; because a larger fraction may be eliminated from the normal tissues before decay occur. Secondly, because of better time to circulate, it causes larger concentrations to diffuse within the tumor tissue and may thereby provide a less heterogeneous tumor dose [9] and thus increase the probability of tumor cell inactivation. Possible negative aspects of using more long long-lived compounds could be that longer circulation time could potentially cause more catabolism and thereby release of potentially toxic free radionuclide from the carrier and an increase in extravasation, etc.

^{223}Ra ($t_{½}$ = 11.4 d), ^{224}Ra($t_{½}$ = 3.6 d) and ^{225}Ac ($t_{½}$ = 10.0 d) are considered among the most promising α-particle emitting radionuclides for use in targeted radionuclide therapy [10-13]. Because the decay take place via several radioactive daughter nuclides, generating three additional alpha-particles for each series, a large amount of energy (26-28 MeV) is released in the form of α-particles when considering the total decay chains of mother and daughter nuclides in the ^{225}Ac, ^{224}Ra and ^{223}Ra series (Table 1). Thus, these radionuclides have the potential to deliver therapeutically relevant doses from a low amount of injected activity, provided the daughter nuclides decay in the same volumes as their mother nuclide.

Generated from long-lived storable sources, these series are beneficial in terms of securing continuous supply to clinics. The ^{223}Ra, ^{224}Ra and ^{225}Ac can be obtained from generator systems based on ^{227}Ac ($t_{½}$ = 21.8 y), ^{228}Th ($t_{½}$ = 1.9 y) and ^{229}Th ($t_{½}$ = 7340 y) respectively. The several days half-lives of ^{223}Ra, ^{224}Ra and ^{225}Ac are in some aspects advantageous, allowing time for the preparation, quality control, and shipment of the radiopharmaceuticals. Care must be taken to avoid radiolysis though, e.g., by using sufficient dilution of the product [14]. A less favorable feature of the daughter nuclides produced in these series is the potential translocalization from the mother nuclides. This would probably be less of a problem with the ^{223}Ra series because 75% of its total alphas are delivered within a few seconds ($t_{1/2}$ ~ 4 seconds) after the ^{223}Ra decay. This problem may be more pronounced with the ^{225}Ac-series, because the ^{225}Ac decays directly to ^{221}Fr, which has a half-life of 4.9 minutes, and are likely to translocate away from its parent. Translocalization could be a significant problem with the ^{224}Ra-

series as well since ^{224}Ra decays directly to ^{220}Rn, a noble gas with a half-life of 56 seconds.

Several studies have recently addressed the development and evaluation of chelators for radioimmunotherapeutic use of actinium [15-22] and radium [23,24], but a bifunctional chelator capable of binding actinium and radium, to e.g. a monoclonal antibody, in a high yield, rendering a product, which is stable *in vivo*, has not yet been demonstrated.

Lipsomes may be used to target therapeutic agents, including radionuclides [25-27]. Preferential tumor accumulation of small (~100 nm) unilamellar vesicles in experimental animal models [28,29] as well as in patients with primary and metastatic disease have been reported [29-31]. Furthermore, polymer coated, long-circulating liposomes [32-34] formulated from long-chained phospholipids and cholesterol have a lipid phase transition temperature above physiological temperature, a low bilayer permeability and low lipid exchange. Such vesicles may therefore be exploited for the encapsulation of α-emitting radionuclides. The primary objective of the present work was to provide a method for preparation of sterically stabilized liposomes (SSL) containing radium and actinium. Because of low availability of ^{225}Ac, ^{228}Ac ($t_{1/2}$ = 6.13 h) was used as the actinium tracer. A second goal of the study was to evaluate the stability of the radio-liposomes in a physiological medium. Lastly, ligand targeting was evaluated for binding to folate receptor expressing cells *in vitro.* Using radio-liposomes coated with a folate-$F(ab')_2$ construct.

2. Materials and methods

2.1. Reagents and equipment

The equipment used for γ-spectrocopy consisted of a high purity germanium detector from Canberra (Meriden, CT, U.S.A) coupled to a multichannel analyzer from EG&G ORTEC (Oak Ridge, TN, U.S.A). The NaI(Tl) well-type detector was from Harshaw Chemie BV (De Meern, Holland). A Beckmann LS 6500 (Beckmann, Fullerton, CA, USA) was used for liquid scintillation counting. Insta-Gel plus scintillation cocktail was from Packard Bio-science, Groningen, The Netherlands. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[Poly(ethylene glycol)$_{2000}$] (DSPE-PEG$_{2000}$), N-{ω-[4-(p-Maleimidophenyl)butanoyl]amino} poly(ethylene glycol) 2000] 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (DSPE-PEG$_{2000}$-MPB), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol were all supplied by Northern Lipids (Vancouver, British Colombia, Canada). The manual extruding device used for downsizing of liposomes was from Avestin (Ottawa, Canada). The 1,4,7,10 tetraazacyclododecane- *N,N',N'',N'''*-1,4,7,10 tetraacetic acid (DOTA) chelator was from Macrocyclics, (Richardson, Texas, U.S.A). Ultrex grade HNO_3 was supplied by J.T. Baker (Phillipsburg, NJ, U.S.A.) and high purity 6 M HCl was from Fisher Scientific (Pittsburgh, PA, U.S.A). Bis-(2-ethyl hexyl) phosphoric acid was from Fluka (Buchs, Switzerland). [^{3}H]-folic acid was obtained as a solution of the potassium salt, 1 % in ascorbic acid, from Amersham Pharmacia Biotech, (Buckinghamshire UK). Dipex-2 extraction chromatographic material was from Eichrom (Darien, IL, U.S.A). 0.22 μm cellulose acetate filters was from Millipore, Bedford, MA, U.S.A). NAP-5 and PD-10 pre-packed size exclusion chromatographic columns

(Sephadex-G25) were from Pharmacia (Uppsala, Sweden). Reagents for the Bio-Rad Detergent compatible protein assay and ion exchange resins were from Bio-Rad (Hercules, CA, U.S.A). 4 Å molecular sieves in dimethyl sulfoxide (DMSO) with a water content less than 0.05 % and barium atomic absorption spectroscopy standard solution were all purchased from Fluka (Buchs, Switzerland). The $^{232}Th(NO_3)_4$ was produced by Merck (Darmstadt, Germany). The sample had been stored for more than 20 y. Other reagents used in this study were obtained from Sigma (St. Louis, MO, USA), including the calcium ionophore A23187 which has a specificity for divalent over monovalent cations.

All water used was ion-exchanged and distilled. The buffer used for the ionophore-mediated cation loading of liposomes were adjusted to the desired pH using L-arginine (free base).

2.2. Detection of radioactivity

^{223}Ra and ^{228}Ac were measured by γ-spectroscopy using an intrinsic Ge-detector. ^{228}Ac was quantified by its 911.2 keV γ-ray (26.6 % probability) and its 968.9 keV γ-ray (16.1 %). ^{223}Ra was quantified by its 269.5 keV (13.7 %) and the 271.2 keV (10.8 %) γ-ray from ^{219}Rn .

Radioactivity measurements of ^{223}Ra were also performed by liquid scintillation counting and by using a NaI(Tl) well-type detector. ^{223}Ra was measured at radioactive equilibrium with its daughters.

2.3. Production of ^{223}Ra

^{223}Ra was produced from a generator based on ^{227}Ac/ ^{227}Th as described elsewhere [35]. Briefly, ^{227}Ac and ^{227}Th were retained in an extraction

chromatographic resin, which allows selective elution of ^{223}Ra, formed from the decay of ^{227}Th, in 1 M HCl. The eluate from the generator was evaporated to dryness and the radioactivity dissolved in 5 mM HCl. The solution was filtered through a 0.22 μm cellulose acetate filter prior to use in the liposome experiments.

2.4. Production of ^{228}Ac

^{228}Ra intended for use as generator material for ^{228}Ac was isolated by solvent extraction from ^{232}Th [36]. By this procedure thorium is selectively extracted into the organic phase while radium remains in the aqueous phase. Briefly, ^{232}Th-nitrate was dissolved in 20 ml of 0.1 M HNO_3 and extracted three times with portions of 70 ml of a 2 M solution of Bis-(2-ethyl hexyl) phosphoric acid (HDEHP) in heptane. The aqueous phase was subsequently washed with 3x30 ml heptane. After this, the aqueous solution was concentrated to 10 ml by evaporation and then applied to a column of 4 mm internal diameter and length of 70 mm filled with Amberlite XAD-7HP resin for removing residual organic compounds. For further purification, the solution containing ^{228}Ra and ^{224}Ra was applied to a 3x40 mm column containing 0.2 g of AG50W-X12 cation exchange resin (200-400 mesh, H^+-form). The column was washed with 10 ml of 1 M HNO_3 followed by the elution of ^{228}Ra, ^{224}Ra, ^{212}Pb and ^{212}Bi with 5 ml of 3 M HNO_3. The ingrowth of ^{228}Ac ($t_{1/2}$ = 6.1 h) from ^{228}Ra reaches equilibrium in a couple of days, but the eluate was stored for one month in order to allow ^{224}Ra to decay.

^{228}Ac were separated from ^{228}Ra on a 3 x 40 mm column of AG50W-X12. ^{228}Ra was eluted in 5 ml of 3 M HNO_3 followed by the extraction of ^{228}Ac with 5 ml of 6 M HNO_3. In preparation for the loading procedure, the solution containing ^{228}Ac was evaporated to dryness and the residue dissolved in 0.1 M HNO_3. The ^{228}Ac produced in this manner contained less than 0.05 % ^{228}Ra as measured by γ-spectroscopy on samples stored for a time corresponding to >10 half-lives of ^{228}Ac.

2.5. Preparation of vesicles

Small unilamellar vesicles were prepared by hydration of a thin lipid film and extrusion [37]. DSPC and cholesterol in a 2:1 molar ratio, typically 20 and 10 μmol respectively, were dissolved in chloroform in a 10 ml round bottom flask. DSPE-PEG_{2000} was included in the preparation at 5 mol % unless otherwise noted.

The solvent was removed by evaporation at reduced pressure and the lipid mixture was kept at < 5 mTorr for at least four hours. The dry lipid film was then hydrated in 0.5 ml of 300 mM citric acid and 25 mM DOTA, at pH 4. The resulting suspension was subjected to five cycles of freezing and thawing using acetone / solid CO_2 and an oil bath set at 65°C. After this followed repeated extrusion through polycarbonate filters employing a manual extruding device at 65 °C. The suspension was filtered 19 times through filters of a pore size of 100 nm. In preparation for the loading of radionuclides, the extra-liposomal aqueous phase was exchanged for 300 mM sucrose, 20 mM 4-(2-hydroxyethyl) piperazine-1-sulphonic acid; (HEPES), pH 7.4, by employing a PD-10 column equilibrated in this medium.

The size and polydispersity of the vesicles was determined by dynamic light scattering using a custom made instrument. The samples were placed in the sample cell and allowed to equilibrate at the measuring temperature. An argon-ion laser (Lexel 95) operating at 514.5 nm wavelength was focused onto the sample and the scattered light was measured at several angles to assure absence of multiple scattering effects. The size and polydispersity index was calculated using the method of cumulants [38]. The analysis of data obtained from light scattering measurements has been described in detail by López-Amanya and Marangoni [39]

The liposome preparations had a mean vesicle diameter of 120 nm and a polydispersity index of 0.4-0.5.

2.6. Loading of ^{223}Ra and ^{228}Ac into liposomes

^{223}Ra-liposomes and ^{228}Ac-liposomes were prepared by ionophore mediated loading of preformed liposomes. The desired quantity of ^{223}Ra or ^{228}Ac in 5 mM HCl was added to a vial containing a film of the Ca-ionophore (corresponding to 0.004 mol % relative to the phospholipid content of liposomes) and pH was subsequently adjusted to 7.4 by adding 300 mM sucrose/ 20 mM HEPES, pH 7.4. Liposomes, typically corresponding to 1 μmol phospholipid, were added and the mixture incubated at 65°C for 30 min unless otherwise noted. The loading of radioactivity was quenched by adding 100 μl of 10 mM ethylenediamine- *N,N'*-tetraacetic acid (EDTA) in phosphate buffered saline (PBS) and the mixture was then left for 10 min. Separation of radionuclide-containing liposomes from radionuclides present in the solution external to the liposomes was done by size-exclusion chromatography [40,41] using a PD-10

column (Sephadex G-25). The lipsomes eluted in the void volume and the radionuclide bound to EDTA eluted in the fraction corresponding to small molecular weight species.

2.7. Stability studies

The liposome suspension was incubated in human serum (Sigma, St.Louis, MO, USA) at 37° C with occasional shaking. The concentration was 1 μmol phospholipid / ml serum. After the desired time period and aliquot was taken and added 100 μl of 10 mM EDTA in PBS, pH 7.4. The leakage of ^{223}Ra or ^{228}Ac was determined by separating the mixture on a column of Separose CL-4B, internal diameter of 20 mm, length of 45 mm, using PBS as eluent.

Separate control experiments were performed to establish the uptake of radionuclides present in serum into liposomes. For these procedures liposomes were prepared and treated in an identical manner as was used for the radiolabeling of liposomes, except for the radioactivity. The liposomes was subsequently applied to a PD-10 column, eluted in PBS and thereafter stored at ambient temperature for 4 hours. After this, the solution containing liposomes was added to serum in to obtain a concentration of 1 μmol lipid / ml of serum. ^{228}Ac or ^{223}Ra in PBS were then added and the mixture incubated at 37° C. After the desired time interval, an aliquot of the solution was added 100 μl of 10 mM EDTA in PBS, pH 7.4 and subsequently applied to Sepharose CL-4B columns. In these experiments, less than 2 % of the added radioactivity eluted in the liposome fraction.

2.8.Preparation of folate conjugated F(ab')$_2$

The antibody fragment used in this study was a human protein F(ab')$_2$ Røv, i.e., a fragment of a myeloma antibody of subclass IgG1 and was prepared by pepsin digestion [41]. The stock solution had a concentration of 14 mg/ml in PBS.

[^{3}H]-folic acid (1 % in stabilizing ascorbic acid) was included as a tracer in the folic acid preparation. The [^{3}H]-folic was added as an aqueous solution of the potassium salt to solid folic acid. The preparation was desiccated at 8 mTorr for at least 2 days, protected from light, and thereafter dissolved in DMSO, with water content of less than 0.05 %, in order to prepare a 20 mM solution. The solution was cannulated into a flask containing activated 4 Å sieves and stored protected from light and in an argon atmosphere for 6-10 h.

The folic acid was activated for coupling to protein by adding 6 mol equivalents of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) to the folic acid solution. The reaction was allowed to proceed for 30 min at ambient temperature. After this, a 40-60 fold molar excess of the activated folic acid was added to protein (5-15 mg/ml in PBS) and allowed to react for 30-60 min. Finally, 0.2 ml of 0.3 M glycine in borate, pH 9 was added to quench the reaction. The high pH obtained in the quenched reaction mixture also provide a liquid medium that is effective for separation of non-covalently bound folate from the folate conjugated to protein via size-exclusion chromatography [42]. Here, the folic-acid (FA) F(ab')$_2$ conjugate was separated from reaction by-products using a PD-10 column pre-conditioned in PBS.

2.9. Determination of folate in folate-antibody conjugate

On a routine basis, the folic acid content in purified protein was determined by liquid scintillation counting of ^3H. The protein concentration was determined by the absorption at 280 nm correcting for the contribution to the absorption of folic acid at this wavelength for which the folic acid absorption was calculated from the molar extinction coefficient (ε_M = 25.820) and the concentration of folic acid obtained from the ^3H-measurements.

As a control for the spectrophotometry/tritium measurement procedure for determination of FA:protein ratios, the methods were cross calibrated via Matrix assisted laser desorption time-of-flight mass spectroscopy (MALDI-TOF) by using a Voyager-DE™ RP (PerSpective Biosys, Wiesbaden, Germany). FA-conjugated $F(ab')_2$ was compared to $F(ab')_2$ using protein concentrations in the range of 10-20 nM in phosphate buffer, pH 7.4. The matrix consisted of 1 mM 3,5 Di-methoxy 4-hydroxycinnamic acid in 50 % aqueous acetonitrile plus 1 % tri-fluoro acetic acid. The shift in molecular mass of the major peak was used to obtain the difference in molecular mass between the FA-conjugate and the non-folated $F(ab')_2$. The average number of folate molecules present on the $F(ab')_2$ was established by dividing the mass-difference to the molecular mass of FA (441 g/mol).

2.10. Preparation of folate-$F(ab')_2$ coated liposomes

FA-$F(ab')_2$ intended for conjugation to liposomes was prepared by incubating FA-$F(ab')_2$ in 1 mM 1,4-Dithio-DL-threitol (DTT) at ambient temperature for 2 h followed by elution on a NAP-5 size exclusion column. The fraction containing FA-$F(ab')_2$ was deoxygenated by bubbling argon through the solution and used immediately for reaction with liposomes.

Separate experiments with [^{3}H]FA-F(ab')$_2$ verifed that the DTT did not lead to release of conjugated FA from protein (data not shown).

The liposomes used for conjugation of FA-F(ab')$_2$ were constituted of DSPC/Cholesterol 2:1 and 5 mol % of DSPE-PEG$_{2000}$-MPB relative to total lipid. The ^{223}Ra-liposomes used in these experiments were otherwise prepared as described above. After the loading step, the reaction mixture was allowed to reach room temperature before deoxygenating the solution with argon. FA-F(ab')$_2$ was added to the solution containing liposomes. The final concentration of phospholipid was 10-12 μmol/ml and the protein concentration was 0.3-0.5 mg/ml. The reaction was allowed to proceed for 2 h at ambient temperature with continuous shaking. The reaction mixture was then applied to a column of Sepharose CL-4B -10 for separation of non-liposome associated protein from liposomes. The FA-F(ab')$_2$ conjugated liposomes were eluted in PBS and stored at 4°C for 12-15 h prior to use in the folate-receptor binding assays .

The phospholipid concentration was determined by the Bartlett phosphorus assay [44]. For determination of protein bound to ^{223}Ra-liposomes two different methods were employed:

1. A radiometric method was used which was based on the addition of a trace amount of iodinated F(ab')$_2$ prior to the reaction with DTT. Here, the protein had in advance been iodinated via IodoGen™ according to the procedure described by Fraker and Speck [45]. Liposome-bound ^{125}I was then measured by a NaI well type detector correcting for the spillover in the respective channels from ^{125}I and ^{223}Ra. Samples of a single nuclide and mixtures of the nuclides were used as reference.

2. As a supplement to the radiometric method, the protein concentration was also determined by the detergent-compatible Bio-Rad protein assay. Prior to this procedure, liposomes were ruptured by the addition of Triton-X100.

The protein concentration was converted to the number of $F(ab')_2$ per liposome by assuming an average area per phospholipid molecule of 0.75 nm^2, [46] which gives $1.2 \cdot 10^5$ phospholipids/vesicle for a vesicle diameter of 120 nm.

To verify the conjugation of FA-$(Fab')_2$, a separate experiment was performed with liposomes without radium or actinium radionuclides. Here, liposomes were reacted with [^{3}H]-FA conjugated $F(ab')_2$ at a protein concentration of 0.5 mg/ml. The FA/$F(ab')_2$ ratio was 2 ± 0.2 and the phospholipid concentration was 2.5 mM. After 2 h at ambient temperature, the FA-$F(ab')_2$ coupled liposomes were isolated by elution on a column of Sepharose CL-4B. ^{3}H-FA- $F(ab')_2$ on the liposomes was quantified by liquid scintillation counting of the chromatographically obtained fractions. For these experiments, the [^{3}H]-FA-$F(ab')_2$/ liposome ratio was 200 ± 15 (mean ± s.d., N= 3).

2.11. Binding of folate-$F(ab')_2$ conjugated ^{223}Ra-liposomes to cells expressing folate receptor

A human ovarian carcinoma cell line, OvCar-3 (ATTC HTB-161) was used as FR-expressing cells. The cells were cultured in 75 cm^2 plastic flasks containing RPMI 1640 supplemented with 10 % fetal calf serum, penicillin, streptomycin and glutamine. 10 days prior to cell harvesting, this medium was replaced with folic acid-free RMPI 1640 medium supplemented with 10 % calf serum. This was done to ensure the expression of FR at the time when the cells were harvested. Harvested cells were added DMSO and frozen at -80 °C.

For use in the binding assays, cells were thawed and immediately thereafter added 1 % bovine serum albumin (BSA) in PBS and washed and centrifuged twice to remove DMSO. Suspensions of OvCar-3 cells in 1 % BSA in PBS were made at a concentration of $2 \cdot 10^6$ cells/ml. 0.125 ml samples of this suspension were added to 2 ml test tubes. The tubes were then added five different concentrations of ^{223}Ra-F(ab')$_2$ FA-liposomes. To determine non-specific binding of liposomes to cells, parallel tubes were added non-folated ^{223}Ra-F(ab')$_2$-liposomes at the same concentrations. The tubes were incubated on a rotating rack for 3 h at 0° C. Subsequently, the cells were washed with 1 % BSA in PBS and centrifuged. The washing procedure was repeated two times. Finally, the cell associated radioactivity and the radioactivity in the washing solutions were measured by liquid scintillation counting.

The specifically bound fraction of ^{223}Ra-F(ab')$_2$ FA-liposomes was determined as follows: The bound fraction of ^{223}Ra-F(ab')$_2$ FA-liposomes ÷ the bound fraction of ^{223}Ra-F(ab')$_2$-liposomes.

Data from the cell binding experiments were used to made binding plots consisting of the concentration of the specifically bound FA-antibody conjugate on the abscissa versus the specific binding / concentration of free conjugate on the ordinate. The binding constant was determined from the slope of the line.

3. Results

3.1. Loading of ^{223}Ra into liposomes

Temperature was found to be an important parameter for the ionophore-mediated uptake of ^{223}Ra in liposomes. (Figure 1). An incubation temperature of 40° C, resulted in less than 2 % incorporation of the added ^{223}Ra after 45 min. By

increasing the incubation temperature to 50° C the yield was improved but was still low compared to the corresponding data obtained at 65 °C. Furthermore, for all incubation temperatures tested, the uptake of ^{223}Ra increased with time up to 45 min, but good yields was also accomplished after only 25 minutes at 65 ^{o}C (Figure 1).

Repeated experiments performed at 65 °C and 30 min incubation time resulted in 78 ± 6 % (mean ± s.d., N= 8) incorporation of ^{223}Ra. Hence, conditions can be defined that result in a rapid, high and reproducible uptake of ^{223}Ra into preformed liposomes.

3.2. Serum stability of ^{223}Ra -liposomes

The results from the serum incubation studies with ^{223}Ra-liposomes are presented in Figure 2. 96 ± 1 % (N = 3) of radium was retained in the liposomes after 24 h and 93 ± 2 % (N = 6) was retained after 100 h in serum. The low fraction of ^{223}Ra released from the liposomes indicates a high stability of the formulation.

3.3. Loading and retention of ^{228}Ac

Good incorporation yield into preformed liposomes was obtained also with ^{228}Ac. Incubating at 65 °C for 30 min resulted in an uptake of 61 ± 8 % (N = 3). Also the retention of ^{228}Ac in liposomes in serum was high, 95 ± 2 % (N = 3) after 24 h (Figure 2).

3.4. Binding of ^{223}Ra- FA-F(ab')$_2$ -liposomes to OvCar-3 cells

Data from the binding assay indicate that there were at least two types of interaction of ^{223}Ra- FA-F(ab')$_2$ - liposomes with the folate receptor (FR). Firstly, a strong binding was obtained at low liposome concentration. A binding constant of the liposome of about 10^{12} was determined from the data points (Table 1). Secondly, at higher liposome concentrations a weaker type of binding apparently occurred. Saturation of the binding sites did not seem to occur within the concentration range tested as demonstrated by the linear increase in binding with increasing concentration of liposomes (Figure 3).

4. Discussion

Systemically administered ^{223}Ra and ^{224}Ra show high and selective bone accumulation [13,47] and may be used for targeting of osseous surfaces. However, to extend the applications of radium isotopes to targeting of soft tissue tumors and for ^{225}Ac to be useful in nuclear medicine, a carrier vehicle is needed. Previously reported studies on actinium [15-22] indicate that there is a need for improvement of actinium conjugates intended for tumor targeting.

Tumor targeting constructs of ^{223}Ra, ^{224}Ra and ^{225}Ac should have the required thermodynamic and kinetic stability to avoid premature release of the radioisotope. In the current study, the retention of radionuclides in liposomes in serum was evaluated. The formulations appeared to be stable with very low release of ^{223}Ra (Figure 2) indicating that the present construct may have a potential as carrier of radium *in vivo.*

Because ^{225}Ac was not available to us at the time when the experiments were done, a preliminary study of actinium with liposomes was done by using

^{228}Ac. The results show that also for actinium a high uptake into preformed liposomes and a high retention with liposomes in serum could be obtained. These results indicate that the liposomes evaluated in the presented study may be suitable as carriers of ^{225}Ac *in vivo* as well.

Although radionuclide-containing liposomes can be prepared by passive incorporation, i.e. by hydrating lipids in an aqueous medium containing the radioactivity, the low solvent capture ratio would cause a low radiochemical yield. Furthermore, this strategy also requires the preparation of a new batch of liposomes for each radiolabeling procedure. In contrast, labeling chemistry based on active loading of preformed liposomes is advantageous because this allows one batch of liposomes to be divided into kits for repeated use in radiolabelings.

In the present study, loading of preformed liposomes was accomplished by using the Ca-ionophore A23187 (Calcimycin), which was shown to efficiently transport actinium and radium into preformed polyethylene glycol (PEG) coated liposomes at 65°C. This ionophore has previously been used successfully to load Ca^{2+} into preformed liposomes in high yield according to Veiro et al [48] and the driving force for the A23187 mediated uptake depended on the intravesicular proton pool rather than on a chelation process. The high yield obtained with Ra^{2+} and Ac^{3+} strongly suggest that conditions have been established which ensure a facile preparation of a new class of radium and actinium radiopharmaceuticals.

For use in targeted radiotherapy, both passively accumulated and ligand targeted liposomes may be considered. It has been shown that passively targeted liposomes accumulate in solid tumor [29, 49]. However, in some animal models, ligand targeted liposomes have been shown to give increased targeting efficacy

and improved therapeutic outcome relative to their non-targeted analogues [25,50]. Due to the short range of and the possibility for a selective irradiation of tumor cells with α-emitters, we decided to evaluate targeting liposomes in the current study. High frequencies of FR over-expression are found in several types of cancer [43] including ovarian carcinoma. In the current study, a novel strategy for preparing FR-targeting liposomes was explored based on linking FA-conjugated $F(ab')_2$-antibody fragments coated onto radiolabeled liposomes. The construct was shown to have a significant receptor mediated binding to FR expressing tumor cells.

The binding constant of FA-$F(ab')_2$-liposomes determined in the present work is within the range 10^9-10^{12} (Table 1). The high-affinity system involved in the cellular uptake of folate has a Ka of ~ 10^{10} [43]. However, the binding constants of ^{223}Ra- FA-$F(ab')_2$-liposomes towards FR were determined in the present study as the binding interaction of conjugates having an average content of more than 300 FA per liposome. Hence it is not unlikely that each liposome can occupy more than one receptor and, for a multivalent construct of this type, the total binding strength is proportional to the product of the individual binding constants.

In contrast to previous folate-conjugated liposomes [51-53], the vesicles in the present study have FA conjugated to $F(ab')_2$-antibody fragments. This strategy represents an opportunity to prepare liposomes possessing affinity towards tumor cells via two different receptors. Furthermore, the use of FA-conjugated mAb reveals the opportunity to prepare a conjugate possessing dual binding affinity. This is possible if the antibody recognizes an antigen different from the FR. Compared to targeted radionuclide therapy with antibodies, the use

of a tumor targeted radionuclide construct possessing dual binding affinity may have advantages because the cellular expression of FR as well as the antigen may vary among the target cells. Sub-populations of cancer cells may therefore exist that do not express one or the other of the receptors. If the antibody itself also binds to the tumor cells via a different receptor, the probability of achieving a therapeutically relevant targeting of tumor cells should be further increased.

It is possible to label the liposomes with folate and antibody by using two independent reaction steps. We chose to combine folate and the $F(ab')_2$ in a construct and subsequently bind this construct to the liposome because (1) this reduced the time needed to prepare the radioliposome, and (2) it facilitates the use of a fixed ratio between folate and the $F(ab')_2$ and (3) the $F(ab')_2$ would act as a spacer for the folate, potentially improving the chances of the folate to react with folate binding proteins on cell surfaces.

The improved pharmacokinetics of recent generations of SSL [54] and the data obtained from the present study indicate that SSL should be further evaluated as carriers of ^{223}Ra, ^{224}Ra and ^{225}Ac *in vivo*. However, with these radionuclides contained in systemically administered liposomes, uptake of a substantial fraction of the radioactivity by the reticuloendothelial system (RES) is likely. The post- metabolic clearance of radium and actinium from RES tissues should therefore be carefully addressed in future studies with these agents. This may include investigations of the effect on clearance from RES tissues via chelating agents contained in the lipsome interior, e.g. DOTA, as was used in the current study. In general DOTA appears to be less suitable as chelator for preparing ,e.g., radioimmunoconjugates based on radium and actinium radionuclides [17,24] but since the chelator concentration is much higher inside

a liposome, chelators like DOTA, with less than ideal stability, may help stabilizing the radionuclides. It should also be noted that at the elevated temperature used for loading of liposomes a more stable complex of DOTA and radionuclide may be generated as observed by McDewitt et al with ^{225}Ac and DOTA [23]. The use of DOTA could potentially also facilitate the loading of radionuclide since when radionuclide has reached the interior of the liposome, the high concentration of the relatively hydrophilic DOTA would cause chelation of the cations in the aqueous interior of the liposome.

The decay-related recoil could potentially separate progeny from a vesicles of a diameter of ~100 nm. The series of ^{223}Ra, ^{224}Ra and ^{225}Ac all yield daughters (^{211}Bi and ^{213}Bi, respectively) which decay by α-emission and have half-lives long enough to allow a biodistribution distinct from the parents and the targeting vehicle. Studies addressing the fate of radium and actinium daughters from liposomes containing these series *in vivo* are therefore warranted to further elucidate the potential of these agents in targeted radiotherapy.

With receptor targeting α-particle emitting therapeutic agents, the specific activity of the compound is important. This is because any unlabeled construct is likely to compete effectively for the receptors on tumor cells. Experiments with various amounts of barium as a loading competitor for radium were performed in this study and the uptake of ^{223}Ra was high (~80 %) even with an amount of barium corresponding to $1 \cdot 10^4$ barium atoms per liposome (data not shown). Based on these results it is indicated that a considerably higher specific activity ^{223}Ra-liposomes can be prepared, if desired, by using the described procedures.

In conclusion, methods have been established that allow actinium and radium to be loaded rapidly and in high yield into preformed PEG-liposomes. The formulations appear to be stable in serum with very low release of the radionuclides studied indicating that the present construct may have a potential as carriers of radium and actinium *in vivo*. In addition, a folate-F(ab')$_2$ coated version of the ^{223}Ra-liposomes have been shown to possess binding properties towards folate receptor expressing tumor cells *in vitro*.

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Table 1.
The decay series from ^{225}Ac, ^{223}Ra and ^{224}Ra

The actinium-225 series	The radium-223 series	The radium-224 series
^{225}Ac (α, 10.0 d)	^{223}Ra (α,11.43 d)	^{224}Ra (α,3.66 d)
^{221}Fr (α, 4.9 min)	^{219}Rn (α, 3.96 s)	^{220}Rn (α,55.6 s)
^{217}At (α, 32 ms)	^{215}Po (α, 1.78 ms)	^{216}Po (α, 0.15 s)
^{213}Bi (β, 45.6 min)	^{211}Pb (β, 36.1 min)	^{212}Pb (β, 10.6 h)
^{213}Po (α, 4.2 μs)	^{211}Bi (α,2.17 min)	^{212}Bi (0.64β + 0.36α, 1.0 h)
^{209}Pb (β, 3.25 h)	^{207}Tl (β, 4.77 min)	^{212}Po (α, 0.3 μs)/ ^{208}Tl (β, 3.1 min) (decay ratio: 0.64 / 0.36)
^{209}Bi (stable)	^{207}Pb (stable)	^{208}Pb (stable)

From Nuclide Explorer data sheets, Institute for Transuranium Elements, Karlsruhe, Germany.
European Commission, Joint Res.Centre, Program Version 1.00 (1999).
Branching, mode of decay and half-life are indicated in brackets.
Branching of less than 2.5% is not included.

Table 2.
Binding constants of ^{223}Ra-FA-Fab'-PEG liposomes binding to OvCar-3 cells [1]

Plot parameter	Binding constant from five liposome /cell ratios[2]	Binding constant from the three lowest liposome /cell ratios[3]
Liposome concentration[4]	$6 \cdot 10^9$	$1 \cdot 10^{12}$
Folate concentration [5]	$2 \cdot 10^7$	$3 \cdot 10^9$

[1] Binding plots were made based on the concentration of the specifically bound conjugate on the abscissa versus the specific binding / concentration of free conjugate on the ordinate. The binding constant was determined from the slope of the line.

[2] The binding constants were estimated from five concentrations of liposomes corresponding to $1 \cdot 10^1$-10^5 liposomes / cell.

[3] The binding constants were estimated by considering only the concentration range of liposomes corresponding to $1 \cdot 10^1$-$1 \cdot 10^3$ liposomes /cell.

[4] The lipid concentration was determined by the Bartlett phosphorus assay (40). The liposome diameter was determined by dynamic light scattering and converted to liposome concentration by assuming an average area of each phospholipid molecule of 0.75 nm^2 which gives $1.2 \cdot 10^5$ phospholipids / vesicle for a vesicle diameter of 120 nm.

[5] The folate / liposome ratio was 360 ± 30 (mean ± s.d., N=3).