

Radiolabeled Pentavalent Dimercaptosuccinic Acid Microspheres for Tumor Therapy: Cytotoxic Assessment and Biodistribution studies

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Abstract

The study focuses on in-vitro ability of Re-188(V) DMSA encapsulated microspheres for blocking the cell growth of U87 MG glioma cell-line and in-vivo biodistribution studies of Tc-99m(V)DMSA microspheres of different sizes and surface property in rats. Tc-99m(V)DMSA and Re-188(V)DMSA encapsulated PLGA microspheres were prepared by solvent evaporation technique. Polyethylene glycol (PEG) was used for coating microspheres to avoid opsonization. MTT Assay and flowcytometry were conducted after incubating Re-188(V)DMSA microspheres (0 mGy-200 mGy) with U87 MG glioma cells for 24, 48 and 120hrs. In-vivo studies were conducted by injecting coated and uncoated Tc-99m(V)DMSA microspheres in normal rats. The results revealed a glioma cell survival rate of 68-95% at 24 hrs. The same was found to be 100-162% at 48 hrs. The observed response at 48 h was due to repairable damage in some cells and excitation of some cells from G₀ phase to G₁, followed by uncontrolled tumor growth. At 120 hrs the survival at different doses was found to be 58-75%. Biodistribution studies showed longer circulation time of PEG-coated microspheres; however, uncoated microspheres were localized in liver and spleen. The bigger microspheres were localized in lungs. Transmission Emission Microscopy (TEM) studies confirmed the localization of microspheres in these organs. This study

indicates that Re-188(V)DMSA microspheres could be used as an alternative to external beam radiotherapy by manipulating the size and surface property.

Key words: Microspheres, Surfactant, MTT Assay, Cytotoxicity, Biodistribution, TEM

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Introduction

Tc-99m(V)DMSA has been used extensively for imaging neurogenic tumors. Re-186/188(V)DMSA is a potential therapeutic analogue of Tc-99m(V)DMSA. The biological system can not distinguish Tc (technetium) and Re (rhenium) and handle them in similar manner as these two share the same group in periodic table (1). The pharmacokinetics of Re-188(V)DMSA (Dimercaptosuccinic acid) have been shown to be identical to that of Tc-99m(V)DMSA (2). At pentavalent state, Tc-99m/Re-188 (V)DMSA, both -SH groups of DMSA are bound with Tc-99m or Re-188 forming a stable complex. Re-188 is a carrier free, generator produced β -emitting radionuclide ($t_{1/2}$ =16.9 h, E_{β} =2.1 MeV) and emits γ photons (155KeV) suitable for imaging by gamma cameras (3). However, if Re-188(V)DMSA, could be delivered to a target tissue/organ in the form of microspheres, it may have the greater therapeutic advantage.

Radioactive microspheres are able to deliver high radiation doses to a target area without damaging the normal surrounding tissues. The effective treatment range in tissue is not more than 12 mm for β -emitters. The emitted β -electrons interact mainly with water, lose energy and lead to activate atoms. The activated species (e.g., free radicals) are responsible for therapeutic effects and damage DNA of cancer cells. Radionuclide-based tumor therapy has been performed mostly with β -particle emitting isotopes, mainly because of their availability and favorable characteristics (4-6). The radioactive microparticles could replace the use of isotopes alone for sustained and targeted therapy (7,8). The target size should match with the radiation range of the radioisotope to maximize the therapeutic effect and minimize toxicity (9).

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The pure β -emitters like P-32 and Y-90 have been used during the last decade and have become the dominant radioactive isotopes for many therapeutic applications. However, it has been shown that a certain amount of low-energy γ -radiation can actually be useful for imaging, either during or after the application of the radioactive microspheres (10,11).

The general radiobiology principles underlying external beam therapy and radionuclide therapy are the same but having some significant differences in the radiobiological effects (12). External beam and brachytherapy emissions are composed of photons, whereas radiations in radionuclide therapy are particulate. The β -particles emitted by Re-188 traverse through matter and lose their kinetic energy, eventually follow a controlled path and stop. Because of their small mass, the recoil energy of the daughter nucleus is negligible. In addition, the linear energy transfer (LET) of these energetic, light, and negatively charged (-1) beta particles are very low (~ 0.2 keV/ μ m) along their path up to a centimeter as they are sparsely ionizing. Consequently, their use as therapeutic agents necessitates the presence of high radionuclide concentrations within the targeted tissue. The long range of each emitted electron produces crossfire and negates the need to target every cell within the tumor. Rhenium-188 radionuclide, used for therapy also releases γ -photons (155 KeV) that do not add significantly to the dose delivered to the target tissue. The radiation damage to the kidney and bone marrow by Re-188(V)DMSA does not pose any obstacle for its application in therapy (13,14).

The biodegradable poly(lactic-co-glycolic) acid microsphere delivery system for Tc-99m labeled DMSA was synthesized and characterized by physico-chemical analysis for delivery of radiation to the tumor site (15,16). Re-188 is a short lived radioisotope, allows shorter time-period for tumor to recover and grow (17,18). The toxic effect of initial burst might be compensated by retention of Re-188(V)DMSA at tumor site. Being the small molecule, Re-188(V)DMSA could easily be cleared from the body (20).

The present study focuses on the in-vitro ability of Re-188(V)DMSA microspheres, when present as a microencapsulated drug delivery system, for blocking the cell growth and to study biodistribution of Tc-99m(V)DMSA microspheres in Albino Wistar rats.

Materials and Methods

The specifications for the principal reagents are as follows: Dimercaptosuccinic acid, poly (lactic-co-glycolic) acid (75:25) (PLGA, MW = 90,000-126,000 g/mol) and poly (vinyl alcohol) (PVA, MW = 30,000-70,000 g/mol). Re-188 was obtained from an alumina-based ^{188}W - ^{188}Re generator provided by Oak Ridge National Laboratory. ^{99}Mo - $^{99\text{m}}\text{Tc}$ generator of Amersham was used for Tc-99m source. Other reagents were of analytical grade. All animal experiments were performed in agreement with the Institutional Animal Ethics Committee, and Atomic Energy Regulatory Board, Govt. of India for injecting radioisotopes in rats. All the animals were disposed through incineration following regulatory limits.

Preparation of Radiolabeled DMSA

Technetium-99m pertechnetate (50 mCi in 0.1ml) was added to 50 mg of lyophilized DMSA powder (containing ascorbic acid and SnCl_2) for the preparation of Tc-99m(V)DMSA. Re-188(V)DMSA was prepared by adding 50 mCi of Re-188 Perrhenate to the DMSA and the solution was then boiled for 30 min at pH 2.0-3.0, subsequently cooled to room temperature. pH of both Tc-99m and Re-188(V)DMSA solutions were raised to 9.0. Radiochemical purity was calculated by instant thin layer chromatography.

Preparation of Microspheres

Tc-99m(V)DMSA and Re-188(V)DMSA encapsulated microspheres were prepared separately using double emulsion solvent evaporation technique (15). Briefly, the oil phase (o) was prepared by dissolving 50 mg PLGA (75:25) or PLGA (50:50) in 3ml Dichloromethane (DCM). Poly (ethylene glycol), PEG (5%w/w) was then added to this solution. Primary emulsion (w_1, o), was formed by mixing the oil phase with Tc-99m(V)DMSA and Re-188(V)DMSA (w_1) followed by homogenization at 10,000 rpm for 3 min. To the primary emulsion, 10 ml of varying concentration of aqueous solution of PVA (10%, and 0.4% w/w) was added (w_2) and homogenized for 4 min at 10,000 rpm to form secondary emulsion (w_1, o, w_2). Emulsion was stirred using magnetic stirrer at room temperature for 3 h to evaporate DCM. The effect of solvent evaporation speed on microspheres was studied by using different speeds of stirring (400 or 1000 rpm). Microspheres were collected by centrifugation at 12,000 rpm for 30 min and were washed thrice with water. The amount of PLGA, DMSA, and temperature were kept constant while varied PVA (surfactant) concentrations were used for preparing microspheres. Microspheres characterization was done by scanning electron microscope (LEO 435 VP, Cambridge, U.K). The size determination and counting was done using Lieca Q-win software (Cambridge, U.K).

Cytotoxicity Studies

U87 MG glioma cells were grown in Dulbecco's Modified Eagles Medium supplemented with 10% (v/v) fetal bovine serum. The glioma cells were used after reaching 90% cell confluency (fourth day) at 37°C in 5% carbon dioxide at 90% relative humidity. Cultures were routinely tested and found to be free from mycoplasma contamination. In-vitro cell survival studies with U87 MG glioma were assayed by mitochondrial activity of live cells. Di-methyl thiazoldiphenyl tetrazolium bromide (MTT) forms insoluble purple colored formazan crystals, the product formed due to metabolic activity of the cells. The formazan crystal formation correlates well with the proportion of live cells in the plate. 3×10^3 cells/well were plated onto 96 well plates. The absorbed dose was calculated and Re-188(V)DMSA microspheres (doses 0 mGy-200 mGy) were added to wells in triplets at an increment of 20 mGy. Three such well plates were prepared and kept for 24, 48 and 120 hrs (5 days). The wells without any added Re-188(V)DMSA microspheres were taken as control.

Cells were subjected to MTT assay. For this, whole media from the wells was taken out and 100 μ l of fresh media was added/well. To this 10 μ l of MTT solution (2mg/ml) was added. The plates were kept in the dark at 37°C in a humidified atmosphere for 4 hrs. After 4 hrs formazan crystals were viewed under light microscope. 100 μ l of DMSO was added to each well for dissolution of crystals

and was immediately read spectrophotometrically at 550 nm with 690 nm as reference in an Anthos ELISA Reader (H-L 1) with untreated cells as blank. The intensity of color developed is directly proportional to the number of viable cells. Percentage survival, for each dose, was calculated.

For flowcytometry studies, the glioma cells were incubated with 140mGy doses of Re-188(V)DMSA microspheres calculated from MTT assay. Re-188(V)DMSA microspheres treated cells were gated on forward and side scattering parameters. The gated populations were analyzed for propidium iodine uptake using fluorescence parameters.

Statistical Analysis

Repeated measure ANOVA was applied to observe the mean significant difference of survival at various point of time (24 hrs, 48 hrs, 120 hrs).

Biodistribution Studies

Wistar rats of 250 ± 25 g body weight were obtained from the Institute Animal House and put under standard laboratory conditions (12h light/dark cycle, ambient temperature $25 \pm 2^\circ\text{C}$) with free access to standard rat chow and water. Each rat was anesthetized with intraperitoneal injection of Na-pentobarbitone (35mg/kg body weight). Rats were injected intravenously (i.v.) through tail vein with 100- μCi of each Tc-99m(V)DMSA microspheres (0.3-1.8 μm), Tc-99m(V)DMSA microspheres (8-16.0 μm) and PEG coated Tc-99m(V)DMSA microspheres (0.3-2.0 μm).

The rats were monitored by planar imaging, using a dual head gamma camera (Millenium VG, G E Healthcare) with low energy all purpose (LEAP) collimator. Five planar whole body images were acquired at 30 min, 1, 2, 6 and 12 hrs after intravenous administration, in order to determine gross distribution of radioactive microspheres. The liver, spleen, kidney, heart, lungs, brain, bladder, bones, intestine and muscles of the rats were removed, washed with ice-cold saline. The weight and radioactivity associated with blood and organs were measured. The results were expressed as a percent injected dose per gram of tissue (%ID/g).

Transmission Electron Microscopy

To investigate the distribution of radiolabeled microspheres in the organs, the transmission electron microscopic (TEM) studies of different organs were performed. Small pieces (1mm) of each organ was fixed in modified Karnovsky's fluid buffered with 0.1M sodium phosphate buffer at pH 7.4 [21]. The specimens were kept in lead shield for the decay of $^{99\text{m}}\text{Tc}$. The specimens were then dehydrated in graded acetone solutions and embedded in CY 212 araldite. Ultra thin sections of 60-80 nm thickness were cut using an ultracut E ultramicrotome. The sections were stained in alcoholic uranyl acetate (10 min) before examination the grids in a transmission electron microscope (Philips, CM-10) operated at 60-80 kV.

Results

Preparation of Tc-99m (V) DMSA/ Re-188(V) DMSA Microspheres

Radiochemical yield of Tc-99m (V) DMSA microspheres and Re-188(V) DMSA was 97% and 95%, respectively. Size of radiolabeled microspheres prepared with 10% PVA (with and without PEG) and 0.4% PVA were in the range of 0.3-

2.0 micron and 8-16 μm respectively. Microspheres were spherical and smooth (Figure 1). For cytotoxicity studies Re-188(V)DMSA microspheres (10% PVA) were prepared and for biodistribution studies of Tc-99m(V)DMSA, microspheres were prepared using 10% PVA (with & without PEG) and 0.4% PVA.

Cytotoxicity Studies

At 24 hrs the percentage survival of glioma cell-line treated with different doses of Re-188(V) DMSA microspheres (0-200 mGy) was between 95-68%. The lowest survival was observed at 140 mGy and highest survival was observed at 180 mGy. The percentage survival of glioma cell treated with different doses of Re-188(V)DMSA microspheres(20-200 mGy) was 100 -162%, showing the overall increased growth as compared to untreated control cells (Figure 2). Highest growth was observed at 120 mGy and lowest growth was observed at 200 mGy. The behavior pattern of cells at 120hrs was similar as observed at 24hrs. The percent survivals of glioma cells, treated with different doses of Re-188(V)DMSA microspheres (20-200 mGy), were 75%-48% with lowest survival at 120-140 mGy (Figure 2). Flowcytometry study also demonstrated the same lowest survival (47-48%) at 120hrs (Figure 3B). From the repeated measures it was found that there was significant difference in cell survival (%) between each time point i.e. 24 with 48 hrs; 48 with 120hrs and 24 with 120hrs ($p < .001$).

Biodistribution Studies

The biodistribution images of uncoated Tc-99m(V)DMSA microspheres(size 0.3-1.8 μm) showed blood pool activity at 30 min. Maximum liver uptake (53% ID/g) was reached at 1hr. Liver counts were decreased only up to 36% ID/g at 12hrs. Rest of the organs showed constantly low activity during the study period (Figure 4). However, the blood pool activity of PEG coated Tc-99m(V)DMSA microspheres of size 0.3-2.0 μm was constantly high (52%-38%) throughout the study i.e. 30 min to 12 hrs (Figure 2).

The images of Tc-99m(V)DMSA microspheres (size 8-16 μm) showed maximum activity (87%) in lungs at 30 min. The lung counts remained high up to 12 hrs. Bladder and kidney showed fluctuating activity. Rest of the organs showed minimal uptake equivalent to background activity (Figure 6). Tc-99m(V)DMSA microspheres were also visualized in the TEM sections of liver, spleen and lung (Figures 7 a,b,c), indicating the presence of microspheres in these organs. Microspheres were not visualized in other organs.

Discussion

The U87 MG glioma cells, when incubated with different doses of a beta emitting Re-188(V) DMSA microspheres, the ionizing radiations of energetic β -particles produced repairable sub lethal damage. The biological clearance of encapsulated Re-188(V) DMSA is controlled by microspheres clearance. Thus, the effective half-life of the encapsulated Re-188(V) DMSA, within the body, will be controlled by microsphere clearance. Moreover, microspheres retention time is increased at tumor site (due to enhance permeability and retention property of tumor), hence Re-188(V) DMSA can be retained for longer duration for effective cell killing (modifying/altering cell survival). Re-188 follows its own physical radioactive

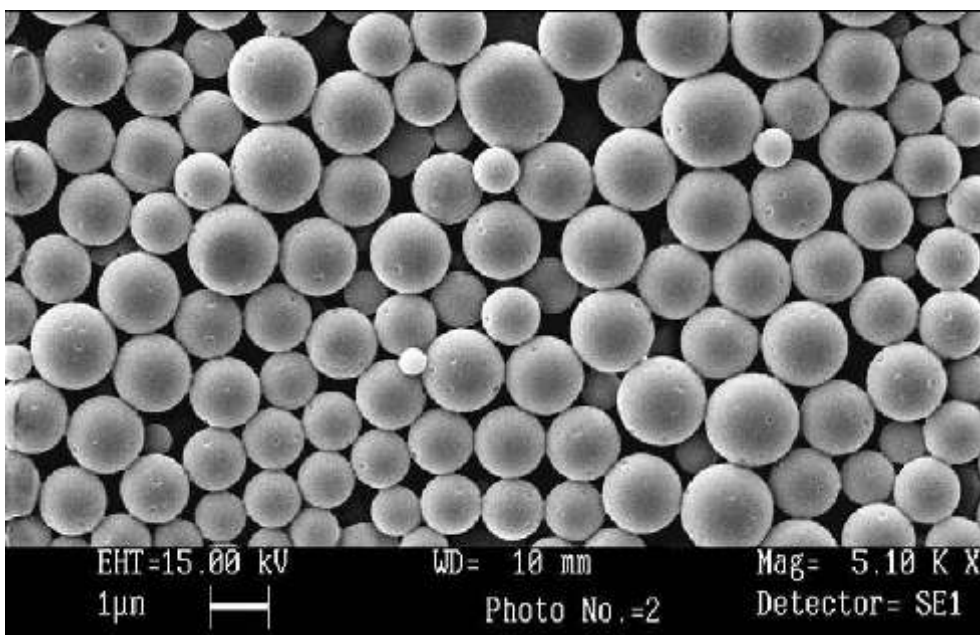


Figure 1. Electron Micrograph (SEM) of Re-188(V) DMSA Microspheres.

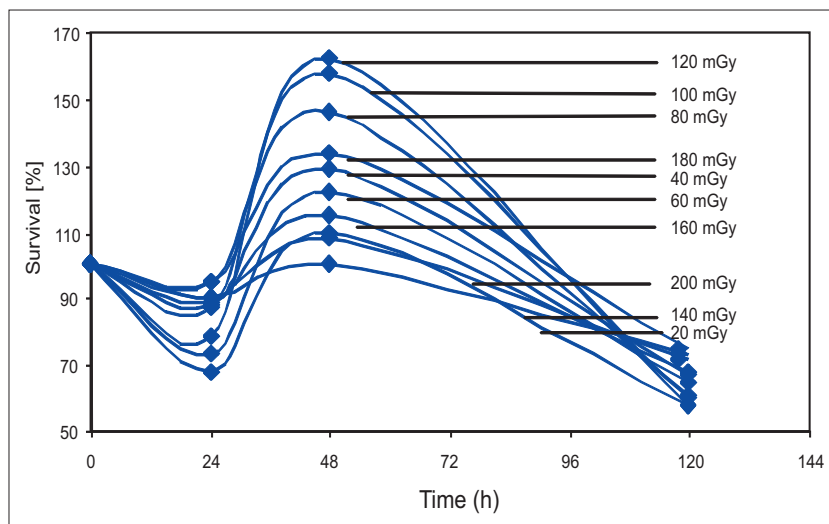


Figure 2. In vitro Cell Survival of U87 MG Glioma Cells Incubated with Re-188 (V) DMSA Microspheres.

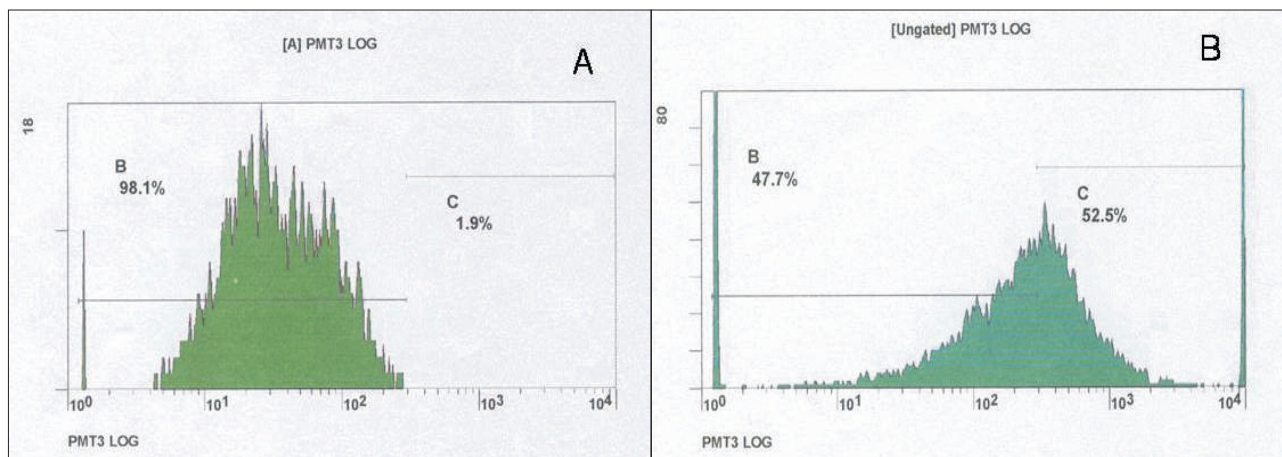


Figure 3. Cytotoxicity Studies using Flowcytometry. (A) Untreated Glioma cells (control) and (B) Glioma Cells Treated with 140m Gy for 120h

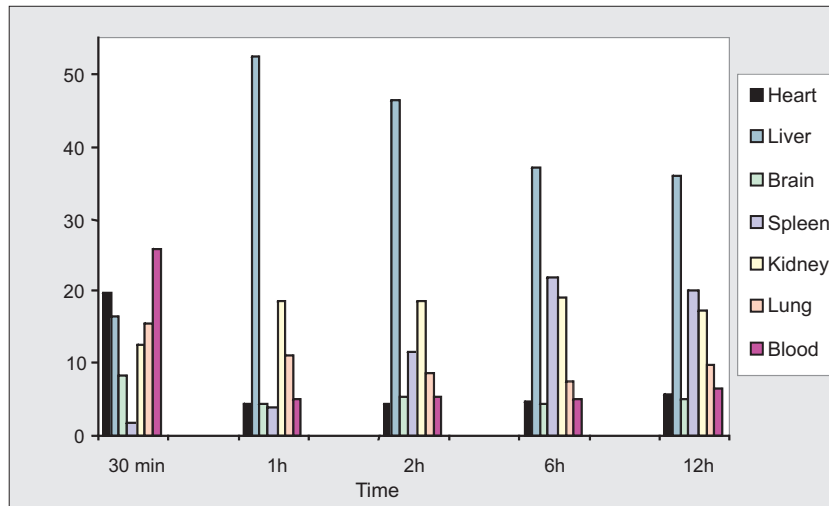


Figure 4. Biodistribution of Uncoated Tc-99m (V) DMSA Microspheres (0.3- 1.8 μm) in Rats (n=3).

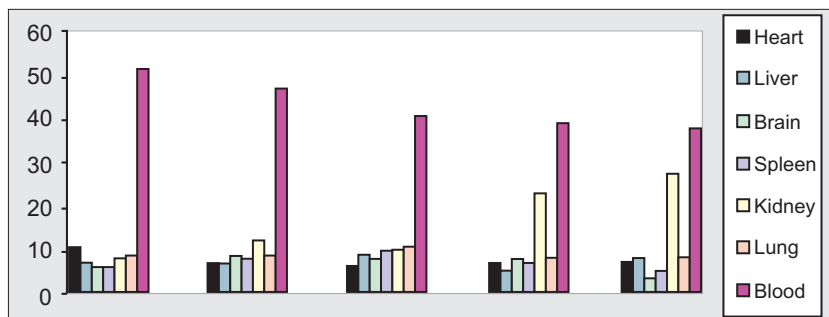


Figure 5. Biodistribution of PEG Coated Tc-99m (V) DMSA Microspheres (0.3-2 μm) in Rats (n=3)

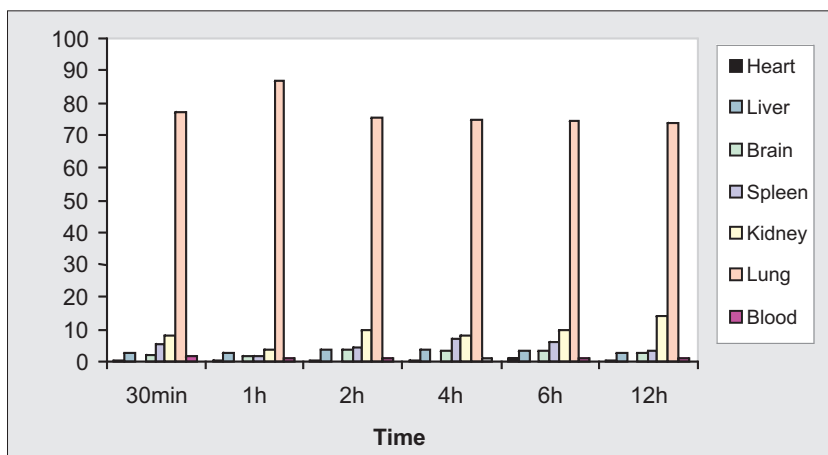


Figure 6. Biodistribution of Tc-99m (V) DMSA microspheres (8-16 μm) in Rats (n=3)

decay properties that will provide dose rate/electron flux in decreasing manner with time. The Cells are not equally radiation sensitive during all phases of the cell cycle (22-25). When the cells were irradiated with Re-188(V) DMSA microspheres, those which were at radiosensitive phase responded and the resultant decline in cell survival as observed at 24 hrs (Figure 2). Some of the cells which might have repairable damage had survived. The repaired tumor cells followed uncontrolled division of growth. Additionally, tumor cells which were earlier in G_0 phase of cell division got triggered into cell division cycle leading to

the overall response observed at 48 hrs. At 120 hrs there was a sharp decline in cell survival as shown in figure 2, which was in agreement with the other published results (26). The results of the flowcytometry study support our findings (Figure 2B).

The biodistribution studies of Tc-99m (V) DMSA microspheres revealed excellent in vivo stability and no uptake was visualised in the thyroid gland and stomach. Normally, the foreign particles when injected, undergo opsonisation (by blood components), which makes them amenable to be detected by the defence system of the body-

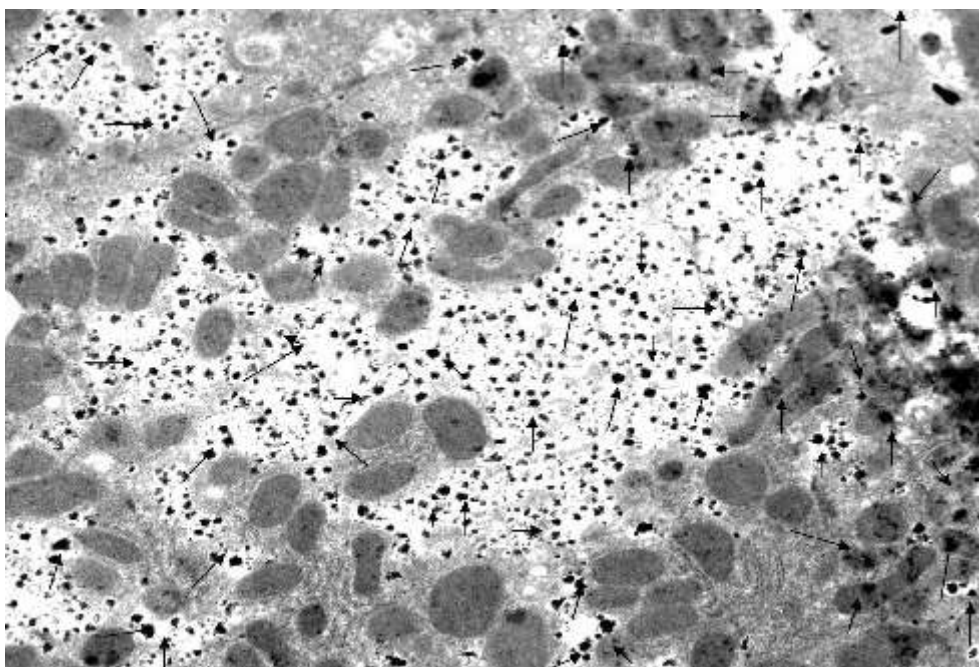


Figure 7a. An electron micrograph of liver loaded with uncoated microspheres of size (0.3-2.0, &) at magnification 1400X

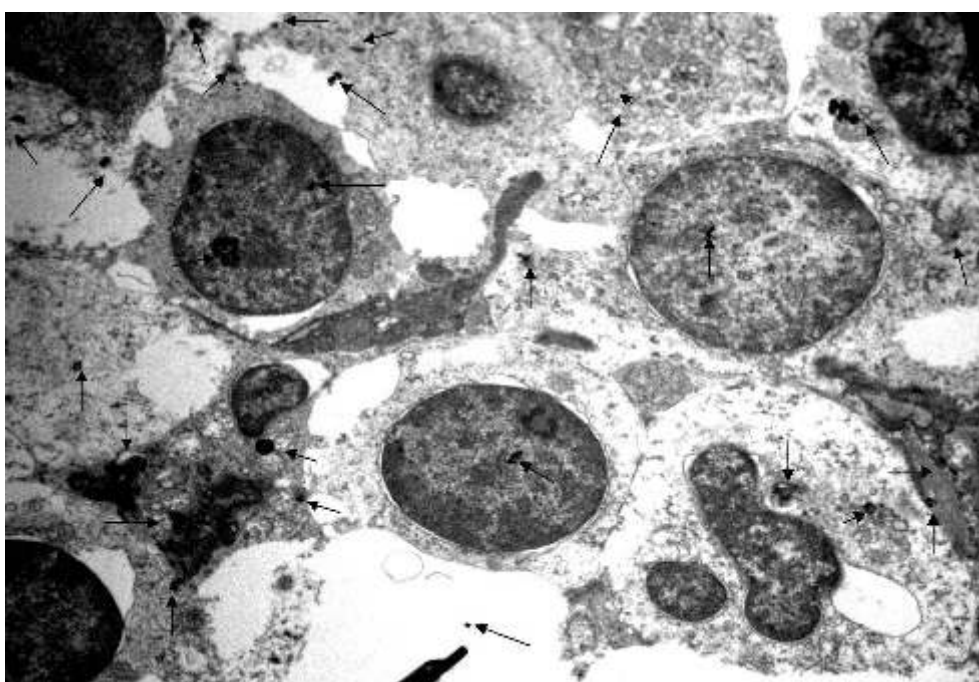


Figure 7b. An electron micrograph of spleen filled with uncoated microspheres of size 0.3-2.0 μm () at magnification 880X

the reticulo-endothelial system (RES). The Kupffer cells of the liver are extremely efficient at removing particles recognized as foreign (27). In the present study, maximum uptake of small microspheres (prepared without PEG coating) in the liver and spleen was reached up to 53% and 28%, respectively (Figure 5). This form of delivery can be of value to treat diseases that involve elements of the RES. Since maximum uptake is in liver and spleen, these microspheres will not be suitable for delivering dose to the organs other than RES. We therefore, prepared microspheres which could deliver dose to other organs also.

The unwanted capture of the colloidal particles by the RES can be avoided by the surface modification of these microspheres by the adsorption on a hydrophilic polymer such as polyethylene glycol (PEG). The PEG layer provided a barrier for adsorption of plasma components on microspheres; as a consequence opsonisation was greatly reduced. PEG coated particles showed extended circulation time. We had already demonstrated that the microsphere sizes were not affected when microspheres were fabricated with PEG coating (15). Small PEG coated microspheres, which remain in the circulation for longer duration, have

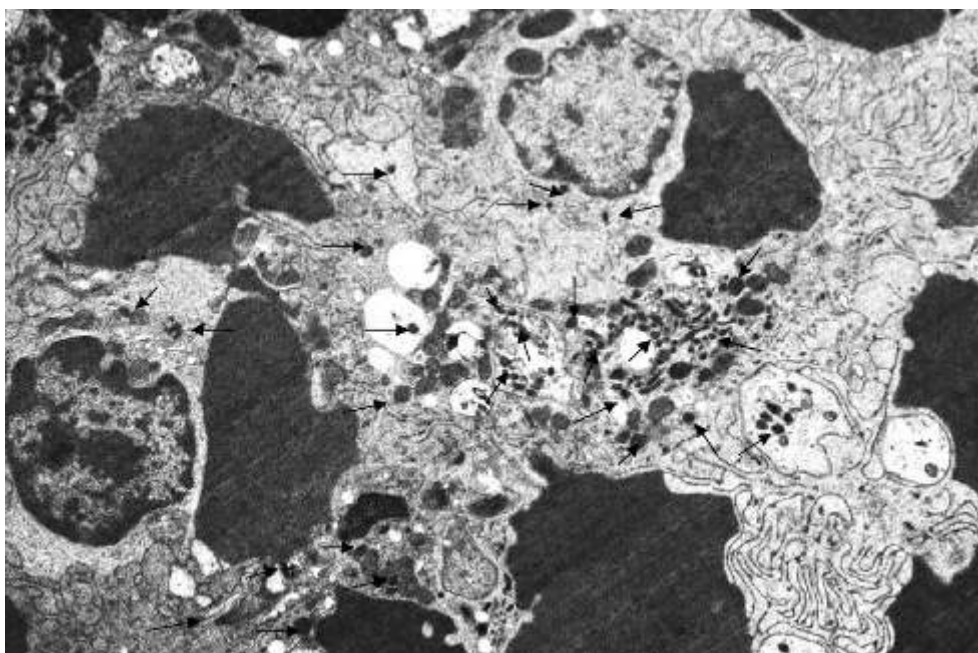


Figure 7c. An electron micrograph of lung filled with microspheres of size 8-16 μm () at magnification 880X

potential for site specific delivery of drugs (12,28). Since the tumour cells have the property of enhanced permeability and retention (EPR), these circulating microspheres will be concentrated in the tumour (29). The present study assessed the rational approaches in the design as well as the biological performance of such microspheres. The PEG coated Tc-99m (V) DMSA microspheres showed long circulating property. The maximum liver uptake reached only 9% as compared to 53% with PEG uncoated microspheres (Figures 4 and 5). Tc-99m (V)DMSA microspheres concentration in blood remained high up to 12 hrs indicating the increased blood residence time of Tc-99m (V)DMSA after encapsulation and PEG coating (Figure 5). The bigger particles could be used to treat lung cancers as these particles were captured by simple mechanical filtration in the lung capillaries. After intravenous injection, the microspheres of bigger size (>9 micron the size of RBC) were effectively trapped in the lungs (Figure 6). Histological studies confirmed the presence of Tc-99m (V)DMSA microspheres in liver, lungs and spleen (Figures 7a,b,c).

Conclusion

Re-188 (V)DMSA microspheres displayed significant effect on cell survival of U87 MG Glioma cell-line. The biodistribution studies showed the trapping of small microspheres in liver and spleen. But after coating with PEG, these microspheres remained in circulation for longer time. Microspheres of bigger size were trapped in lungs. Re-188 (V)DMSA microspheres could be used for site specific targeted radiation therapy of some tumours by manipulating the size and surface properties.

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