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^{188}Rhenium(V)-dimercaptosuccinic acid loaded poly(lactic-co-glycolic)acid microspheres for targeted radiotherapy: production and effectivity

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Received July 13, 2004, accepted October 19, 2004

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Pharmazie 60: 583–587 (2005)

Poly(lactic-co glycolic)acid (75 : 25) (PLGA) microspheres for the delivery of a radiation dose to tumors were synthesized after loading the ^{188}Re(V) labeled DMSA. The $^{188}ReO_4^-$ in place of $^{99m}TcO_4^-$ was replaced to label DMSA, because of their structural and chemical similarities and to make the molecule site specific for radiotherapy. The radiolabeling efficiency of DMSA was more than 97% as confirmed by ITLC. A solvent evaporation technique was used to encapsulate DMSA in PLGA microspheres. Electron microscopy demonstrated the microspheres size ranged between 0.4–1.8 μm. As demonstrated by DSC, DMSA was encapsulated (20–30%) within the microspheres in solid solution, metastable molecular dispersion or crystallization forms. *In vitro* release studies confirmed the stability of DMSA.

1. Introduction

DMSA is a water-soluble dithiol (containing two sulfhydryl, or −SH, groups) used as an antidote for chelating during heavy metal poisoning. DMSA has a large therapeutic window, and low toxicity, making it superior to other chelating agents available (Aposhian et al. 1983, 1992). It is reported that one of the sulfhydryl groups in DMSA binds to a cysteine residue of albumin, leaving the other −SH group available to chelate metals (Aposhian et al. 1986; Miller 1998). DMSA forms a complex with pentavalent technetium (^{99m}Tc(V) DMSA) at alkaline pH. This radiopharmaceutical has been used extensively to localize a number of tumor types (Ohta et al. 1984a, 1984b, 1988). In the pentavalent state both the −SH groups of DMSA are coordinated with the ^{99m}Tc molecule, forming a complex that is identical to $[^{99m}TcO(DMSA)_2]^-$ (Blower et al. 1991). ^{99m}Tc(V) DMSA is metabolized within the tumor cells and hydrolyzed to phosphate (PO_4^{3-}) like $^{99m}TcO_4^-$ ion (Horiuchi et al. 1986; Yokoyama 1981, 1985 et al.). ^{188}Re is an analogue of ^{99m}Tc (Blower et al. 2000), which is a beta emitter ($E_{max} = 2.2$ MeV) having appropriate radiotherapeutic properties with a physical half-life of 17 h. It also emits gamma photons (155 KeV) suitable for gamma scintigraphy (Bisunadan et al. 1991). Being in group VII A, the crystal radii of Tc (VII), Re (VII) are 0.51 Å, and 0.52 Å, respectively, whereas the radii of Re(0) and Tc(0) in the free metals are identical. The occurrence of Tc and Re in the same periodic group ensures that their general chemistries are qualitatively similar (Deutsch et al. 1986). On the basis of these facts, it can be reasonably anticipated that analogous Tc and Re complexes will have the same size, shape, dipole moment, charge, ionic mobility, lipophilicity, etc. These complexes when exposed in a dilute aqueous solution, a biological fluid, or on a receptor site, the physical properties of analogous Tc and Re complexes will be indistinguishable, and the environment will handle them in an identical manner (Deutsch et al. 1986). The pharmacokinetics of ^{188}Re(V) DMSA have shown to be similar to that of ^{99m}Tc(V) DMSA (Blower et al. 1998). Microspheres based on the copolymer of lactic acid and glycolic acid (PLGA) has been extensively investigated in the past primarily due to their commercial availability, versatility, biocompatibility and hydrolytic degradation into resorbable, harm-less products (Kronenthal 1975; Lewis 1990). Drug release from PLGA microspheres mainly depends on the composition of the co-polymer. Many drugs have been successfully incorporated into PLGA microspheres (Castelli et al. 2000; Chandrashekar and Udupa 1996; Tuncay et al. 2000; Ertl 1999; Mu and Feng 2001; Mohr et al. 1999; Birnbaum et al. 2000). Considerable research has been done in evaluating PLGA delivery systems, especially microsphere preparation, drug stability, and release characteristics (Couvreur et al. 1997; Wise et al. 1976). Controlled release systems are of great interest for the treatment of cancer, where improved treatment efficacy, site-specific administration and reduced adverse side effects are of main concern. The biodegradable polymeric delivery system, capable of providing a sufficient and localized radiation dose of radiotherapeutic drug along with some chemotherapeutic agents to the tumor site (Edulund and Albertsson 2002), can also be used as an alternative to external beam radiotherapy. Intra-arterial embolization of unresectable tumors and metastases with radioactive biodegradable microspheres is considered as an effective targeted treatment modality.^{166}Holmium labeled poly (L-lactic acid) (^{166}Ho PLA) microspheres have been especially

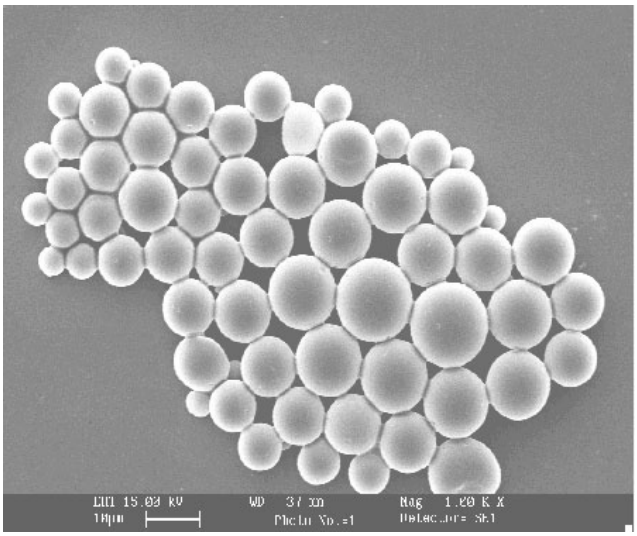

Fig. 1: SEM of microspheres

developed for radioembolization (Nijsen et al. 1999; Van Es et al. 2001) but leaching of free isotope (^{166}Ho) into urine and faeces was the main concern.

The present study focuses on the *in vitro* ability of ^{188}Re(V) DMSA when present as a microencapsulated drug delivery system for blocking the cell growth and potentially enhancing the radiation sensitivity of cells. The concept behind the use of a biodegradable carrier is to develop a radiolabelled delivery system at the defect site. In combination with radiation therapy, it is proposed that the site-specific β-radiation (emitted from ^{188}Re) from the device will reduce the number of remaining tumor cells. It is thought that the localized release of ^{188}Re may eliminate the systemic side effects associated with i.v. administration and provide direct exposure of ^{188}Re to tumor cells. The advantage of a microsphere-based carrier system is that localized radiation dose of drug will be delivered to tumor sites to eradiate cells. This kind of a localized delivery system may eliminate the toxic side effects that are accompanied with systemic administration of radiation doses. The advantage of using ^{188}Re(V) DMSA over isotope alone encapsulated microspheres was that ^{188}Re(V) DMSA has a high affinity for tumor cells and is stable in vivo (Blower et al. 1998).

The object of this study was to (a) characterize the delivery system, and (b) determine the physico-chemical analysis for delivery of radiation to the tumor site. With this aim, a poly(lactic-co-glycolic)acid microsphere delivery system for labeled and unlabeled DMSA was developed.

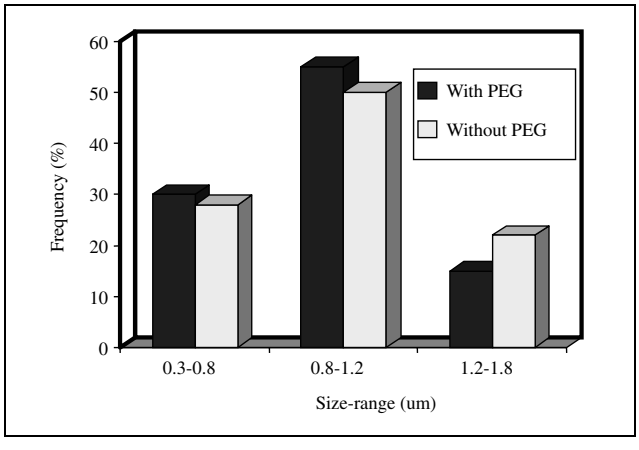

Fig. 2: Histogram of microspheres with and without PEG

2. Investigations and results

2.1. Microsphere characterization

Microspheres were in fine spherical shape with smooth surfaces and without any aggregation or adhesion (Fig. 1). The microspheres remained smooth and spherical in shape even after 25 days in normal saline. The histogram of microspheres is shown in Fig. 2. Additionally, shapes of microspheres were also examined using transmission electron microscopy (TEM) after staining microspheres with 1% phosphotungustic acid (PTA). Microspheres took on negative stain with PTA (Fig. 3).

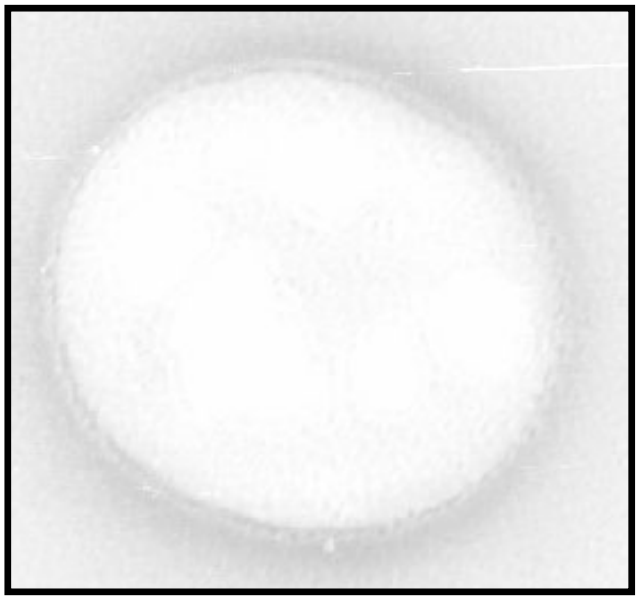

Fig. 3: TEM of microspheres using 1% Phosphotungustic acid

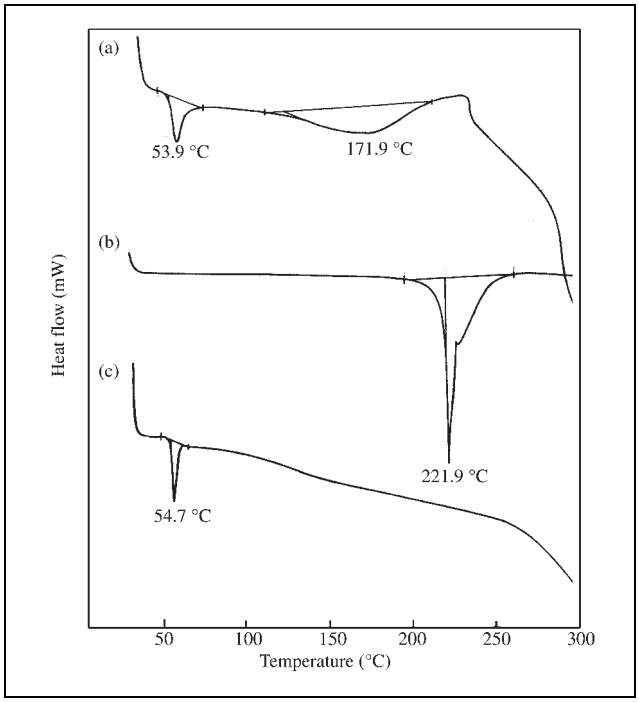

Fig. 4: DSC of microspheres – a) PLGA, b) DMSA, c) DMSA loaded microspheres

2.2. Thermal characterization

Differential scanning calorimetry (DSC) analysis of DMSA (drug), PLGA, microspheres and the drug inside the polymer matrix was carried out. The DSC scan of PLGA showed a melting endotherm at 53.9 °C, while the melting endotherm of DMSA was obtained at 221.9 °C (Fig. 4). In the DSC scan of DMSA loaded microspheres only an endothermic transition at 54.7 °C was observed. Thermogravimetric analysis (TGA) of PLGA (Fig. 5) shows that the polymer was stable up to 250 °C and started losing mass above this temperature. From the TG trace of various samples, the initial decomposition temperature (IDT), the temperature of maximum rate of mass loss (T_{max}), the final decomposition temperature (T_f) and the residual weight (char yield) at 400 °C (%) were recorded.

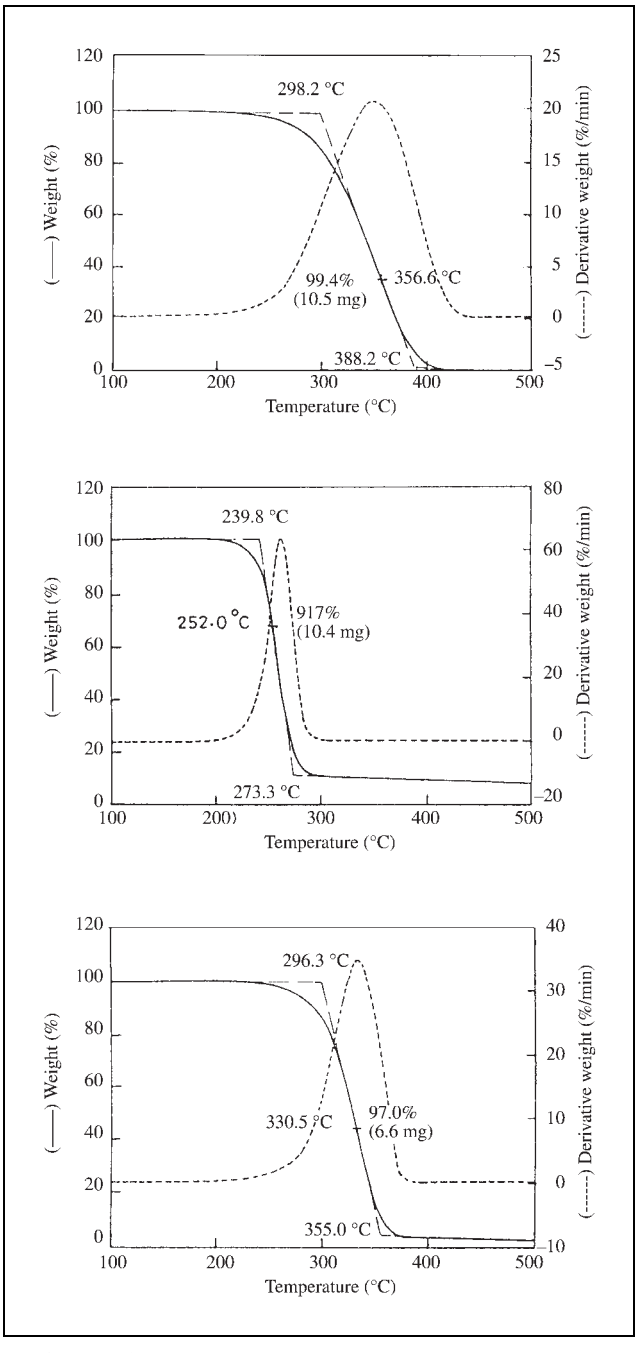

Fig. 5: TGA of a) PLGA, b) DMSA, c) DMSA loaded microspheres

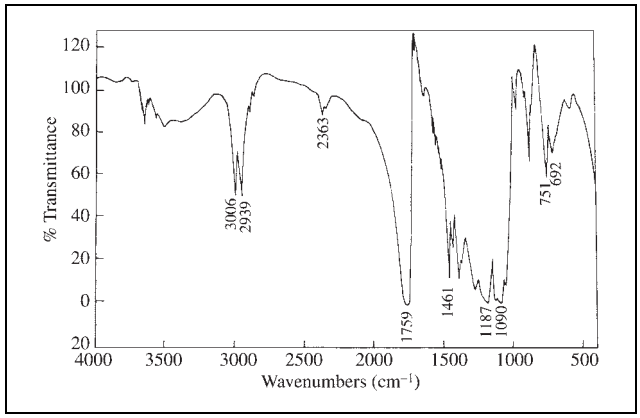

Fig. 6: IR spectra of microspheres loaded with DMSA

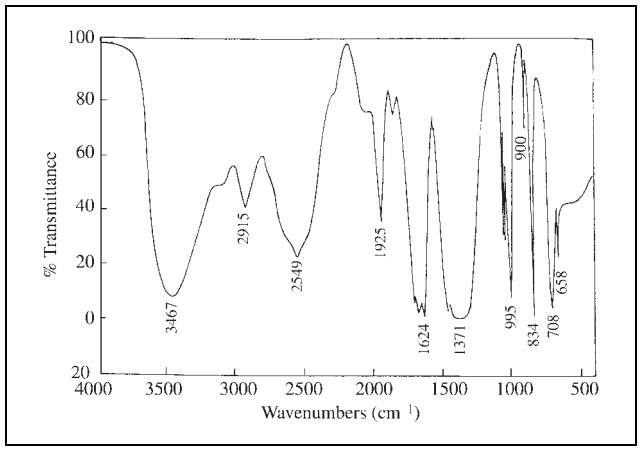

Fig. 7: IR spectra of microspheres loaded with ^{99m}Tc(V) DMSA

2.3. Infra-red analysis

In the IR spectrum of PLGA (75 : 25), DMSA, and of DMSA-loaded microspheres prominent stretching vibration at ~2900–3000 cm^{-1} (−CH), ~1750 cm^{-1} (C=O), ~1080 cm^{-1} (−O−CH_2), ~3500 cm^{-1} (−OH), and ~3000 cm^{-1} (COOH) were observed. A sharp peak of −SH at 692.6 cm^{-1} was present in DMSA (Fig. 6). The IR spectra of ^{99m}Tc(V) DMSA, and of released material from ^{99m}Tc(V) DMSA loaded microspheres are similar. The peak ~995 cm^{-1} corresponds to Tc = O (Fig. 7).

2.4. In vitro release analysis

The microspheres were prepared with and without PEG. For the studies on the release of ^{99m}Tc(V) DMSA from the PLGA microspheres, the loaded microspheres were placed in phosphate-buffered saline and release was determined spectrophotometrically at $\lambda = 410$ nm (Fig. 8).

3. Discussion

Microspheres are smooth and spherical for a long period of time (Fig. 1), indicating the utility of microspheres in radiation delivery. The microspheres were heterogeneous. However, the histogram of microspheres is shown in Fig. 2. Size varied from 0.4 μm to 1.80 μm. In the formation of DMSA-loaded microspheres by the double emulsion solvent evaporation technique, the use of surfactant is necessary to stabilize the dispersed-phase droplets and to inhibit coalescence. The amphiphilic surfactants align

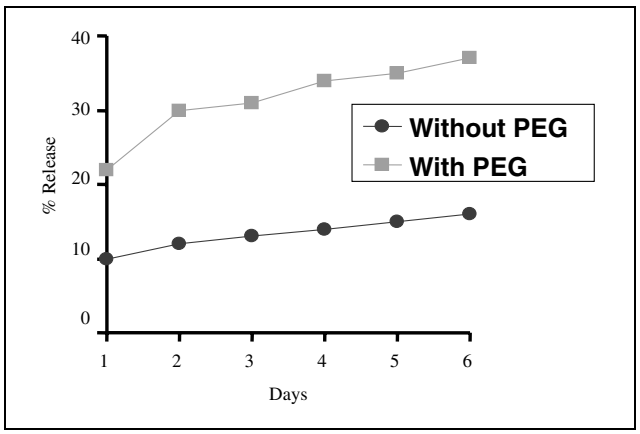

Fig. 8: Release study of microspheres prepared with and without PEG

themselves at the droplet surface, thereby promoting stability by lowering the free energy at the interface between the two phases and resisting coalescence and flocculation of the microspheres. Surfactants employed in the oil/water process tend to be hydrophilic in nature, and among them, PVA is the most widely used and would appear to be the most effective for the formation of microspheres (Arshady 1990). The DSC of microspheres did not show the peak corresponding to DMSA but showed significant release, indicating that the drug may be present in solid solution, metastable molecular dispersion or crystallization and may display relevant properties during *in vitro* release (Mu and Feng 2003). TGA indicated (Fig. 5) that the PLGA polymer was stable up to 250 °C. The char yield was 0.57% in PLGA and 8.26% in DMSA. The DMSA loaded PLGA microspheres had a char yield of 2.95%. These results clearly show the incorporation of 20–30% of DMSA in the microspheres. A small absorption peak was present at 691.7 cm^{-1} indicating that drug (DMSA) was present in the microspheres (Fig. 6). The IR spectrum of ^{99m}Tc(V) DMSA, and of released material from ^{99m}Tc(V) DMSA loaded microspheres showed peak at ~995 cm^{-1} corresponding to Tc = O (Fig. 7). This indicated that the released material is ^{99m}Tc(V) DMSA and ^{99m}Tc(V) DMSA remained intact inside the microspheres and did not lose its chemical identity. For radiation therapy, microspheres should be leak proof, which is practically not possible. We chose PLGA in which lactide concentration is three times than glycolide (3 : 1), which is a comparatively slow degrading polymer. In multiple samples, an initial burst was observed which depended on the presence or absence of PEG in the microspheres. In absence of PEG, the initial burst release was significantly lower (Fig. 8). A possible explanation may be that PEG is water-soluble and may diffuse out during fabrication, creating voids in the microspheres. The microspheres are prepared by the w/o/w method and water-soluble drugs show a significant tendency to migrate towards the aqueous dissolution medium, subsequently concentrating at the surface of the microspheres and increasing the burst effect (Huang and Brazel 2001). ^{99m}Tc(V) DMSA was originally designed as a metabolic mimic of phosphate, able to localize in the cancer and yield the phosphate like the TcO_4^{3-} ion by hydrolysis within the cancer cells (Yokoyama et al. 1985; Blower et al. 2000). Due to enhanced permeability and retention (EPR) property of cancer cells microspheres might have entered inside the glioma cells (Maeda et al. 2000). The pentavalent technetium DMSA core is stable in plasma until delivered to the target site at which the hydrolysis to $^{99m}TcO_4^{3-}$ could occur. As $^{99m}TcO_4^{3-}$ behaves like the phosphate ion (PO_4^{3-}), it interferes with the biochemistry of ATP (adenosine tri phosphate) formation, which is the ultimate source of energy for growth. If, in the cell, energy is depleted growth of tumor could be checked. Hence $^{99m}TcO_4^{3-}$/$^{188}ReO_4^{3-}$ may behave like targeted growth inhibitors. Keeping this point in mind, we studied the release kinetics for up to 25 days. Data showed a sustained release of ^{99m}Tc(V)/^{188}Re(V) DMSA from the microspheres (Fig. 8). Based on these results, the microsphere system could be fabricated as a carrier for therapeutic radioisotope delivery by simply replacing ^{99m}Tc with a therapeutic isotope, the beta emitter ^{188}Re. Enhanced permeability and retention property of the tumor might serve as driving force for microspheres to enter the tumor cells. DMSA serves as a bifunctional drug. First, ^{188}Re(V) DMSA itself is a tumor directed radiopharmaceutical and second, it is a good carrier for isotope delivery (>95% binding). Being a tumor targeted radiopharmaceutical, the ^{188}Re(V) DMSA released during the initial burst, will also accumulate in the tumor. *In vitro* and *in vivo* studies with the therapeutic isotope (^{188}Re) are needed for further successful development.

4. Experimental

Dimercaptosuccinic acid, poly(lactic-co-glycolic)acid (75 : 25) (PLGA, MW = 90,000–126,000 g/mol), poly (vinyl alcohol) (PVA, MW = 30,000–70,000 g/mol), PEG (MW = 5000 g/mol) propidium iodide was from Sigma-Aldrich, ^{99}Mo–^{99m}Tc generator from Amersham for ^{99m}Tc source and ^{188}Re was obtained from an alumina-based ^{188}W-^{188}Re generator from Oak Ridge National Laboratory.

4.1. Radiolabelling

DMSA (50 mg) was dissolved in 0.5ml of bicarbonate solution (7.5% v/w), 15 mg ascorbic acid, 25 mg $SnCl_2$ was added. pH was adjusted to 8.0–9.0 with 1 N NaOH and lyophilized.

4.2. ^{99m}Tc(v)DMSA

50 mCi of pertechnetate was added to 30 mg of above lyophilized sample ITLC was performed after 10 min for quality control. Counts were taken by well counter.

4.3. ^{188}Re(v)DMSA

50 mCi perrhenate was added to 30 mg of lyophilized sample and was boiled for 30 min and cooled to room temperature. ITLC was performed and counts were taken by gamma well counter.

4.4. Microsphere preparation

Microspheres were prepared with different amounts of labeled and unlabeled DMSA and different surfactant concentrations (PVA). DMSA loaded microspheres were fabricated by using the double emulsion solvent evaporation technique (Chen et al. 1998) with some modifications. Briefly, PLGA (75 : 25) was dissolved in 5 ml methylene chloride (oil phase, o). Primary emulsion was formed by mixing and homogenizing (water/oil, w_1/o) at 10,000 rpm for 2 min. To the precold primary emulsion 15 ml, (10%) poly (vinyl alcohol) was added (water phase, w_2) and homogenized for 4 min at 10,000 rpm to form the secondary emulsion (w_1/o/w_2). The emulsion was stirred for 3 h at room temperature to remove the methylene chloride. Microspheres were collected by centrifugation at 10,000 rpm for 10 min. Microspheres were washed with distilled water and resuspended in 1 ml of distilled water and lyophilized. The isolated microspheres were kept in desiccators until further use. Different batches of microspheres were prepared with DMSA, ^{99m}Tc(V) DMSA, and ^{188}Re(V) DMSA. Microspheres were prepared using PEG with PLGA and without PEG. The size and initial burst of microspheres prepared were compared.

4.5. Microsphere characterization

Surface morphology and average particle size of microspheres were examined by scanning electron microscopy (SEM) of unlabeled DMSA loaded microspheres. Microspheres were affixed to an SEM stub, coated with gold

and examined under a scanning electron microscope (LEO 435 VP), operated at 15–25 KV. Microspheres suspended in distilled water, incubated for different time intervals were visualized under SEM.
Transmission electron microscope (TEM) measurements were carried out using a microsphere suspension containing 1% (w/v) phosphotungustic acid and placing on a carbon film coated on a grid. The specimen on the copper grid was not stained. A 60 KV Philips CM10 TEM was used for this purpose.

4.6. Thermal studies

Thermal characterization was done using a TA 3100 thermal analyzer equipped with a 910 DSC module and a 951 TG module. For DSC studies 3–4 mg of the sample were loaded in a hermetically sealed cell and the measurements were taken over a temperature range of 30–350 °C at a heating rate of 10 °C/min under static air atmosphere. For TG studies a sample mass of 8 ± 2 mg and a heating rate of 10 °C/min was used under nitrogen atmosphere (flow rate 60 ml/min).

4.7. Infra red spectroscopy

IR spectra of PLGA, DMSA, and DMSA loaded microspheres; ^{99m}Tc(V) DMSA and released material of ^{99m}Tc(V) DMSA were recorded in KBr pellets using a Nicolet 460 ESP Protégé FTIR spectrometer. IR of ^{99m}Tc(V) DMSA microspheres and released material from these microspheres was done after physical decay of ^{99m}Tc(V) DMSA.

4.8. In vitro ^{99m}Tc(V) DMSA release

^{99m}Tc(V) DMSA microspheres (10 mg) were studied for release of DMSA by suspending in phosphate buffer saline (PBS) at pH 7.4. The vials were fixed horizontally in a shaker at 37 °C, and samples were withdrawn at regular time intervals after centrifugation at 8000–10000 rpm for 10 min. The removed volume was replaced by fresh medium. Supernatant was analyzed spectrometrically for ^{99m}Tc(V) DMSA release. Spectrometric analysis was done at $\lambda = 410$ nm (Blower et al. 1991) with a UV-160 A (Shimadzu) spectrophotometer.

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