JPP Journal of Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY

JPP Journal of Pharmacy and Pharmacology

JPP 2011, 63: 1401–1410

Received November 17, 2010
Accepted April 18, 2011
DOI
10.1111/j.2042-7158.2011.01306.x
ISSN 0022-3573

Research Paper

Targeted lung cancer therapy using ephrinA1-loaded albumin microspheres

Hung-Yen Lee[a,b], Kamal A. Mohammed[a,c], Shriram Peruvemba[a,c], Eugene P. Goldberg[b] and Najmunnisa Nasreen[a,c]

[a]Division of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, [b]Biomaterials Center, Department of Materials Sciences and Engineering, University of Florida and [c]NF/SG Veterans Health System, Malcom Randall VA Medical Center, Gainesville, FL, USA

Abstract

Objectives EphrinA1, the ligand of EphA2 receptor tyrosine kinase, has been proven to suppress the growth of tumours. The aim of this study was to conjugate ephrinA1 on the surface of albumin microspheres and investigate the non-small cell lung carcinoma growth and migration *in vitro*.

Methods Bovine serum albumin microspheres were designed and synthesized using a natural polymer albumin by emulsification chemical cross-linking. EphrinA1 was then conjugated on the surface of microspheres by imine formation. The microspheres conjugated with ephrinA1 (ephrinA1-MS) were characterized for particle size, surface morphology, loading efficiency and stability *in vitro*. The ephrinA1-MS were labelled with fluorescein isothiocyanate to determine phagocytosis. In addition, the effects of ephrinA1-MS on A549 cell growth and migration were determined.

Key findings Albumin microspheres exhibited low toxicity for A549 cells (above 90% cell viability). More than 80% of microspheres were phagocytosed within 2 h of incubation. EphrinA1-MS decreased the expression of focal adhesion kinase more effectively than recombinant ephrinA1 alone. Furthermore, ephrinA1-MS showed significant inhibition of non-small cell lung cancer migration when compared with resting cells. EphrinA1-MS attenuated the growth of tumour colonies in matrigels.

Conclusions The developed ephrinA1-MS may serve as potential carriers for targeted delivery of the tumour suppressive protein ephrinA1, with minimal cytotoxic effects and greater antitumour therapeutic efficacy against non-small cell lung cancer.

Keywords albumin microspheres; ephrinA1; intratumoural lung cancer therapy; non-small cell lung cancer; receptor-targeted drug delivery

Introduction

Lung cancer, primarily non-small cell lung cancer (NSCLC), is the leading cause of cancer-related mortality; about 30% of all cancer deaths are owing to NSCLC.[1] The 5-year survival rate for all lung cancer cases is about 16% and this extremely high rate of mortality has not significantly improved despite new drug development and clinical research. One new procedure, intratumoural chemotherapy, has been explored clinically in recent years. This technique involves the direct intratumoural injection of a cancer drug using a bronchoscopic needle-catheter and is termed endobronchial intratumoural chemotherapy. Clinical studies have shown endobronchial intratumoural chemotherapy to be remarkably effective in reducing tumour burden, with relief of endobronchial obstruction and symptoms of dyspnea.[2–5] Recently, to improve the effectiveness of intratumoural chemotherapy, various drug-loaded microspheres have been designed to prolong very high intratumoural drug concentrations and further minimize any risk of systemic toxicity.[6,7] In the current study, albumin microspheres (AMS) were used as carriers for in-vitro delivery of the antitumour agent ephrinA1.

Microspheres can be defined as solid, approximately spherical particles ranging from 1 to 1000 μm in size.[8] Microspheres prepared from a variety of synthetic polymers and biopolymers have been widely studied for their application in cancer treatment.[9,10] Among the various biodegradable particulate drug carriers available for consideration, we regard the most abundant natural plasma protein, serum albumin, as a most appealing biocompatible carrier for drug delivery.[11,12] Furthermore, the abundant functional groups on the surface of

Correspondence: Najmunnisa Nasreen, Division of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, University of Florida, PO Box 100225, Gainesville, FL 32610, USA.
E-mail: nnasreen@medicine.ufl.edu

bovine serum albumin (BSA) facilitate physisorption and covalent coupling to functional polymers, proteins and biomolecules.[13] In previous animal studies, we demonstrated the effectiveness of drug-loaded AMS for the treatment of a Lewis lung carcinoma and a mammary adenocarcinoma.[14] The drugs or bioactive molecules incorporated into microspheres were active on the microsphere surface and released from the microspheres by diffusion and by degradation of the BSA matrix. Furthermore, the release of loaded bioactive molecules from the microspheres is controllable as a function of particle size, particle size distribution, AMS cross-link density and degradation rate, and the method of binding to the microsphere matrix.

Protein delivery using nano- and micro-sized microspheres based on various biodegradable polymers, including poly(lactic-co-glycolic acid), lecithin and biocompatible hydrogels, has attracted significant research interest in recent years.[15–18] Proteins have been loaded in the microsphere matrix, physically adsorbed on the surface and covalently conjugated with reactive functional groups on the particle surface.[19–23] However, protein delivery using microsphere particles can be complicated by protein denaturation during preparation, especially for some ionic polymers.[24] In this regard, AMS may prove more effective as a stabilizing the matrix for adsorbed or conjugated proteins.

The Eph transmembrane tyrosine kinases are the largest family of receptor tyrosine kinases. Among this family, EphA2 receptor tyrosine kinase was found to be overexpressed in aggressive cancers and most NSCLCs, but it was not expressed in normal tissue.[25–27] Recently, Brannan *et al*.[28] reported that more that 90% of NSCLC patient samples overexpress receptor EphA2 and, moreover, the expression of EphA2 was detected in all the NSCLC cell lines tested. It has been shown that by binding to the receptors on the cell membrane with a glycosylphosphatidylinositol anchor, ephrinA1 was found to inhibit the proliferation and migration of lung tumour cells due to downregulation of EphA2 receptor expression.[29] Thus, the recombinant ephrinA1Fc can target only EphA2 in NSCLC and suppress tumour growth and invasion for NSCLC treatment and prevention. In addition, the specific target of ephrinA1 to EphA2 may reduce systemic toxicity and conserve the adjacent normal lung tissue.

In this study, we synthesized 5- to 10-μm AMS by a water/organic solvent dispersion procedure developed in our laboratory. This particle size was selected based on our previous research which indicated that microspheres were especially effective for intratumoural injection and tumour perfusion. EphrinA1 was conjugated to AMS by imine coupling of ephrinA1 amino groups to free aldehyde groups that remain on AMS after glutaraldehyde cross-linking. The structure and composition of ephrinA1-MS were characterized and antitumour activity was measured using A549 human NSCLC cells.

Materials and Methods

Cell lines and reagents

The A549 human lung adenocarcinoma epithelial cell line was obtained from the American Type Cell Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Sigma, St Louis, MO, USA) containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (100 μg/ml), 0.25% D-glucose, 0.2% sodium bicarbonate and 1% sodium pyruvate. The recombinant mouse/Fc was purchased from R&D Systems (Minneapolis, MN, USA).

Synthesis of AMS and loading of ephrinA1

The AMS were synthesized following the method of Longo *et al*.[11] In brief, 5% w/v solution of cellulose acetate butyrate (CAB) (butyryl content 16.5–19.0%; Sigma, St. Louis, MO, USA) in 1,2-dichloroethane (DCE) (certified ACS grade, Fisher, Pittsburgh, PA, USA) was used as the continuous organic phase. CAB/DCE solution (16.0 ml) was added to a 50-ml polystyrene centrifuge tube. Then, 1 ml of a 10% w/v aqueous solution of BSA (Sigma) was added into the continuous phase. EM grade glutaraldehyde (16% w/w) was added to BSA through DCE into the emulsion solution. After crosslinking, the AMS were washed and the appearance of microspheres was recorded using an optical microscope (Olympus, Center Valley, PA, USA).

About 1 mg of fresh 16% glutaraldehyde crosslinked AMS was dispersed in 50 μl of phosphate-buffered solution (PBS) containing 50 μg of ephrinA1 in a 1.5-ml centrifuge tube. The tube was placed on a shaker at room temperature for 4 h and centrifuged. The supernatant was removed and the loaded AMS were washed twice in 1 ml acetone. The ephrinA1-MS were air-dried and stored at –4°C until further use. EphrinA1-loaded AMS were stored as dry powder at –20°C for longer-term storage (more than 6 months) to prevent degradation from hydrolysis.

To demonstrate the stable conjugation between ephrinA1 and AMS, the AMS were loaded with fluorescein isothiocyanate (FITC) labelled ephrinA1 and observed using a fluorescence microscope. The ephrinA1 was labelled using a FluoReporter FITC labelling kit (Invitrogen, Eugene, OR, USA). After the loading process, the FITC-loaded AMS were vortexed vigorously in PBS to remove excess unconjugated ephrinA1. The samples were washed in PBS by repeated centrifugation. The microspheres were incubated in PBS for 8 h and washed in PBS before observing under a fluorescence microscope.

Particle size distribution of AMS

The particle size distribution of AMS in PBS was determined by a Coulter LS 13320 laser diffraction particle size analyser (Beckman Coulter, Inc., Brea, CA, USA). PBS was used as the suspension fluid and AMS were dispersed in PBS and sonicated before measurement. The laser obscuration range used was between 8 and 12% during measurement.

Surface morphology of ephrinA1-MS

The ephrinA1-MS were coated with a thin layer of gold/platinum and mounted on a metal stub. The surface morphology of AMS was investigated by scanning electron microscopy with a JEOL JSM 6400 (JOEL, Tokyo, Japan).

Cytotoxicity and phagocytosis of AMS by A549 NSCLC cells

The FITC-labelled AMS were prepared by using the mixture of 90% BSA and 10% albumin-FITC conjugate. Cells

(1×10^6) were seeded in each well of a 24-well culture plate and incubated in media containing various concentrations of FITC-labelled AMS. After the desired incubation time, the media were discarded and the cells were washed using PBS until excess AMS were removed. The cells were then trypsinized, fixed in paraformaldehyde and counted using a hemocytometer under a fluorescence microscope.

Longterm cytotoxicity of AMS was determined by using the CytoTox-ONE homogeneous membrane integrity assay (Promega Corp., Madison, WI, USA). Cells (5000) were seeded in each well of a 96-well plate and incubated in media containing various concentrations of AMS. The release of lactate dehydrogenase (LDH) was measured after 6, 24 and 48 h. The LDH release was given in fluorescent units and each experiment was done in triplicate.

In-vitro release rate and loading efficiency of ephrinA1

The release rate of ephrinA1 from the ephrinA1-MS was determined with 10 μg/ml lysozyme (egg white; Fisher) in PBS. In a 15-ml centrifuge tube, 1 mg of ephrinA1-MS was dispersed in 10 ml of PBS containing 10 μg/ml lysozyme to simulate the actual concentration of lysozyme in serum. The tube was placed on a rotator and incubated at 37°C. After 1, 2, 3, 5 and 7 days, 1 ml of dispersion solution was collected and filtered using centrifugal filters with 0.1 μM hydrophilic membrane (Ultrafree-CL; Millipore, Billerica, MA, USA). The flow-through was saved for the determination of ephrinA1 release. The concentration of released ephrinA1 in the dispersion solution was measured by an enzyme-linked immunofiltration assay (ELIFA) following the manufacturer's recommendations (Pierce Biotechnology, Rockford, IL, USA). A nitrocellulose membrane was sandwiched between a 96-well sample plate and a vacuum chamber in the ELIFA system. The sample solutions containing released ephrinA1 were slowly pulled through the membrane by vacuum leaving the ephrinA1 on the membrane. Then the reconstituted antibody, anti-mouse (goat IgG; R&D systems), and secondary antibody were added and allowed to filter through the membrane. Finally, the substrate solution was added and the flow-through was collected in a 96-well microplate. The absorbance was measured by using an ELISA plate reader. To distinguish the release rate of ephrinA1 conjugated on the surface from that loaded in the matrix, another batch of ephrinA1-MS was vortexed vigorously to wash off the ephrinA1 loaded in the matrix before being dispersed in PBS solution.

Western blot analysis

Cells were cultured on 60-mm Petri dishes to 80% confluence and then incubated with serum-free RPMI-1640 media containing free recombinant ephrinA1 and ephrinA1-MS. After desired time periods, the media were removed from dishes and each dish was washed with PBS. The cell lysates were scraped in PBS and then centrifuged. The supernatant was discarded and the cells were lysed in radioimmunoprecipitation buffer. To determine the focal adhesion kinase (FAK), proteins were resolved and detected by Western blot using anti-FAK antibody (Cell Signaling, Boston, MA, USA) following a previously reported method.[29]

Cell migration assay

To measure the cell migration, 1×10^5 cells were seeded into each chamber of a four-chamber glass slide. After 48–60 h of incubation, when cells reached 100% confluence, the cultures were scratched by 200-μl pipette tips to create a wound on the cell monolayer. The cell cultures were washed gently in PBS to remove cell debris before incubation in 10% fetal bovine serum containing media with and without recombinant ephrinA1 or ephrinA1-MS. The wound areas were photographed every 6 h and each experiment was done in triplicate.

3D tumour growth assay

Matrigel (BD, Franklin Lakes, NJ, USA) was diluted with serum-free media in a 1 : 1 ratio (matrigel to media). Diluted matrigel solution (200 μl) containing free recombinant ephrinA1 or ephrinA1-MS was added to each well of a 24-well plate and the gel was allowed to set at 37°C for at least 30 min. After the gel was set, A549 cells at a density of 5000 cells per well were plated in 300 μl of serum-free media. Media were changed every 3 days. To monitor the tumour growth, digital pictures were taken of each well every 2 days until the tumour colonies were formed. To eliminate biased judgment, tumour colonies were photographed by two different investigators at different times by randomly choosing three fields in each well. Thus, photographed pictures were blinded and provided to a third investigator for unbiased judgment and a representative photograph selected from each group was provided.

Statistical analysis

SigmaStat 3.5 (SYSTAT Software, Inc., San Jose, CA, USA) was used for statistical analysis. Data are expressed as mean ± SEM. Comparisons were made using the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. The unpaired Student *t*-test was used where applicable. Differences were considered significant at values of $P < 0.05$.

Results

Synthesized AMS size distribution

Albumin microspheres were synthesized and the particle size distribution was determined by volume percentage in PBS (Figure 1). The particle size was controlled over the range of 2–10 μm and about 60% of the microspheres were 5–10 μm. Only around 8% of the microspheres were smaller than 2 μm. The highest point of the size distribution was 5.35 μm and the mean particle size was 4.2 μm. The photomicrograph of the AMS confirmed the particle size distribution as determined by a laser diffraction system. Significant numbers of the AMS were over the size range 4–6 μm, with some small microspheres of around 1–2 μm.

Cytotoxic effects of synthesized AMS

The cytotoxic effects of synthesized AMS in A549 cells was determined by a cell viability assay after 48 h of incubation with various concentrations of AMS (0.10–1.0 mg). Greater

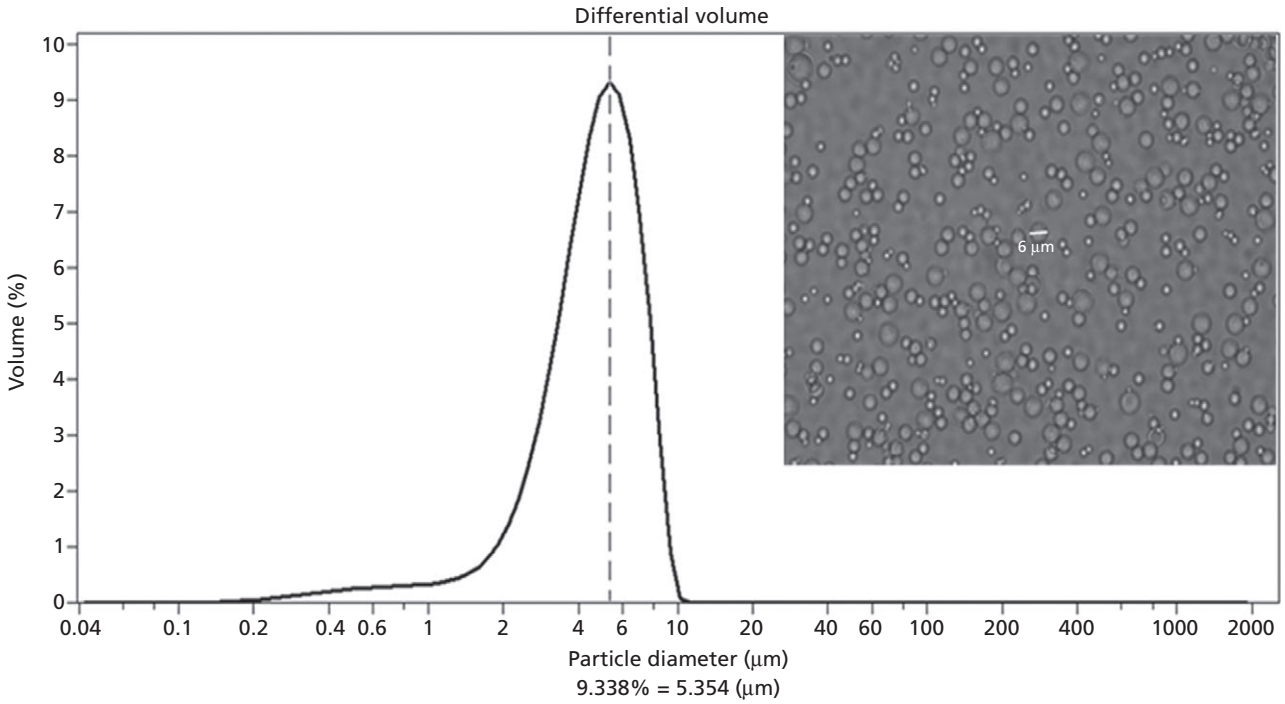

Figure 1 Particle size distribution of synthesized albumin microspheres. Particle size distribution in phosphate-buffered solution was measured using a laser diffraction particle size analyser. About 60% of the microspheres were 5–10 μm. The mean particle size was 4.2 μm and standard deviation was 1.8 μm. The median for the particle size distribution was 4.7 μm and the mode was 5.4 μm. Particle size was further confirmed by an optical microscope.

Table 1 Cell viability of albumin microspheres in A548 cells after 40 min of incubation

AMS concentration (mg/ml)	Cell viability (%)
0	95.7 ± 8.2
0.10	97.1 ± 9.4
0.25	95.9 ± 8.6
0.50	91.9 ± 8.1
0.75	87.6 ± 8.3
1.00	77.6 ± 6.7

AMS, albumin microspheres. Values are mean ± SE ($n = 3$).

than 95% of cells were viable at up to 0.5 mg of AMS. At higher concentrations (0.75 and 1.0 mg/ml) the viability was 87.6 ± 8.3 and 77.6 ± 6.7%, respectively (Table 1). AMS phagocytosis was measured by incubating the A549 cells for 40 min. The phagocytic rate increased with increasing concentration of AMS while maintaining high cell viability (Figure 2a). We also determined the phagocytosis in A549 cells over time using a single concentration (0.25 mg/cm^2) of AMS. At 2 h after incubation, about 80% of A549 cells phagocytosed the AMS, and after 4 h greater than 90% of cells phagocytosed the AMS. The cells cultured in medium alone served as a control (Figure 2a). The cytotoxicity of AMS in A549 cells was determined by LDH assay. The LDH leakage from cells cultured in different concentrations of AMS for 6, 24 and 48 h is shown in Figure 2b. At 6 h of incubation with 0.1 mg/cm^2 AMS, cells showed very low LDH leakage, comparable with cells incubated with media alone. AMS at a concentration of 0.2 mg/cm^2 also showed similar levels of LDH leakage. At 24 and 48 h, cells incubated with AMS showed a slight increase in LDH leakage with increasing AMS concentration. However, the values were not significant when compared with cells alone. This data suggests that the synthesized AMS have low cytotoxic to A549 cells.

EphrinA1 conjugation on AMS

Albumin microspheres were conjugated with FITC-labelled ephrinA1, rinsed with PBS and then incubated in PBS to determine the ephrinA1 conjugation. The conjugated AMS was determined by fluorescence microscopy and FITC labels were clearly observed, implying stable conjugation between ephrinA1 and AMS and that the conjugated AMS were stable in PBS (Figure 3a).

The scanning electron microscopy images of conjugated AMS showed uniform spheres and a smooth surface morphology. The particle size of most conjugated AMS under scanning electron microscopy was 5–10 μm; a few around 2 μm were also noticed in the field. The particle size and surface morphology of AMS did not change after ephrinA1 conjugation (Figure 3b and 3c). This data suggests that the synthesized ephrinA1-MS were stable.

Release rate of ephrinA1 from microspheres

The data in Figure 4 demonstrate that about 5 μg of ephrinA1 was released from 1 mg ephrinA1-MS (matrix loaded and surface conjugated) in 24 h. The release of ephrinA1

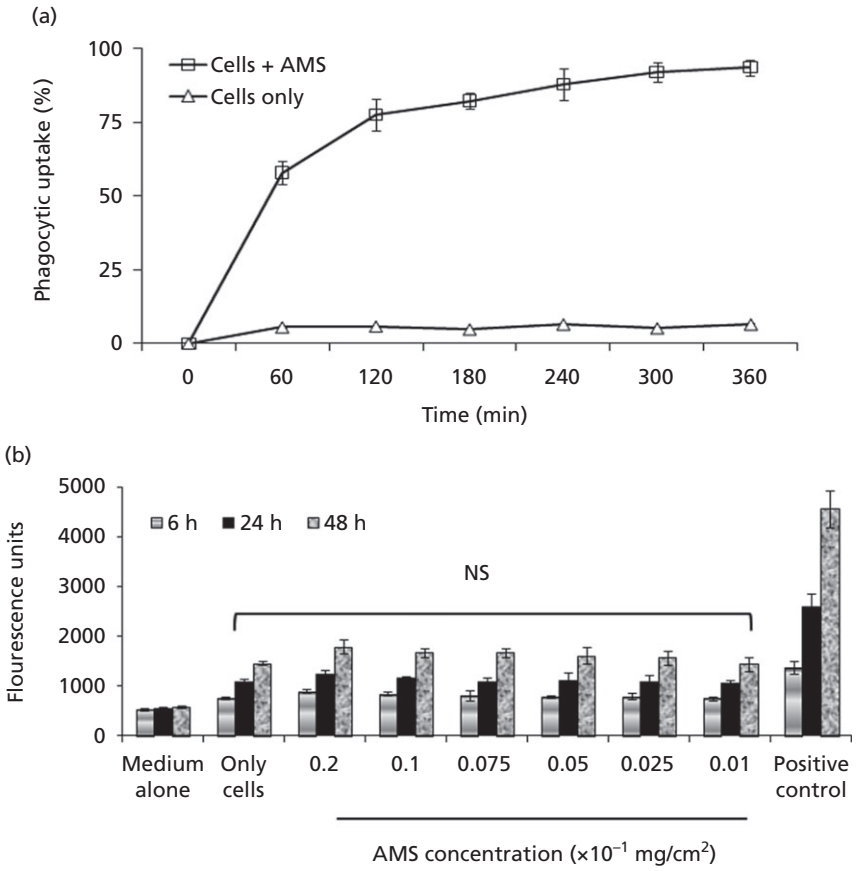

Figure 2 Effect of synthesized ephrinA1-loaded albumin microspheres on phagocytosis and toxicity in A549 cells. (a) Over 80% of albumin microspheres (AMS) were phagocytosed by cells within 3 h at an AMS concentration of 0.25 mg/cm^2. The data presented are mean ± SEM of three independent experiments. (b) AMS showed low cytotoxicity to A549 cells within 2 days. Cells cultured in phosphate-buffered solution alone used as vehicle. The cells treated with lysis solution were used as a positive control. The data presented are mean ± SE of three independent experiments. NS, not significant.

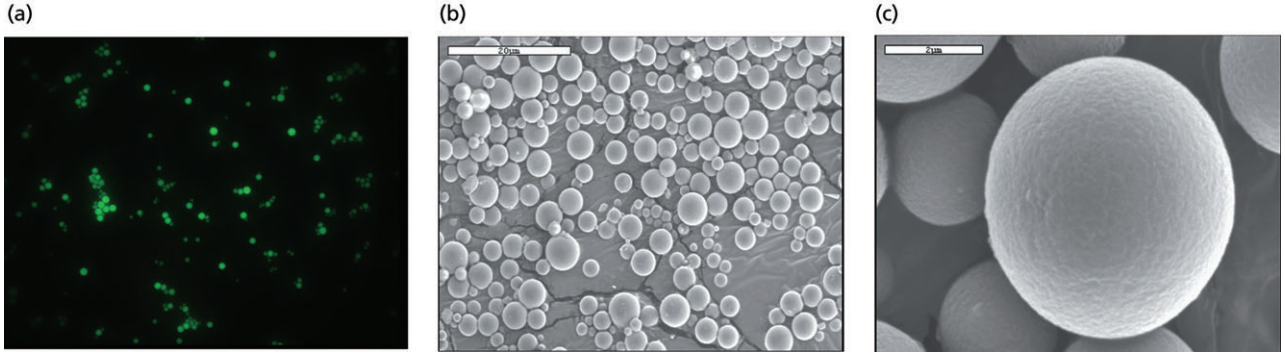

Figure 3 Surface morphology of ephrinA1-loaded albumin microspheres. (a) EphrinA1 was labelled with fluorescein isothiocyanate and then conjugated on albumin microspheres. After conjugation, the microspheres were washed with phosphate-buffered solution and then incubated in phosphate-buffered solution at 37°C for 8 h. The image was taken after incubation using a fluorescence microscope. (b,c) Surface morphology of scanning electron microscopy images of conjugated albumin microspheres.

accumulated to more than 8 μg over 7 days of incubation. After 14 days, the release amount reached about 10 μg, which was assumed as the total loading amount of ephrinA1 in ephrinA1-MS (10 μg of ephrinA1/mg microspheres). Pre-washed ephrinA1-MS (surface conjugated only), showed lower release compared with matrix loaded microspheres over time. From the difference between ephrinA1-MS with and without prewash, it was revealed that about 10% of loaded ephrinA1 was in the matrix and most of the ephrinA1 was conjugated on the surface of AMS.

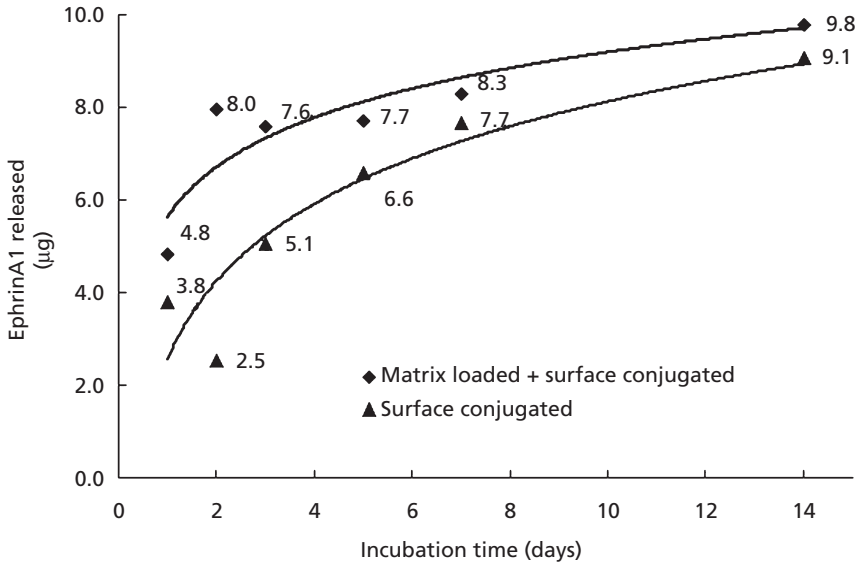

Figure 4 EphrinA1 release rate from albumin microspheres. This figure shows the release of ephrinA1 in a 10-ml phosphate-buffered solution suspension containing 1 mg ephrinA1-loaded albumin microspheres and 10 μg/ml lysozyme. EphrinA1 loaded in the matrix provided fast release to give a high concentration at the beginning, whereas the ephrinA1 conjugated on the surface provided a slower and continuous release into the environment.

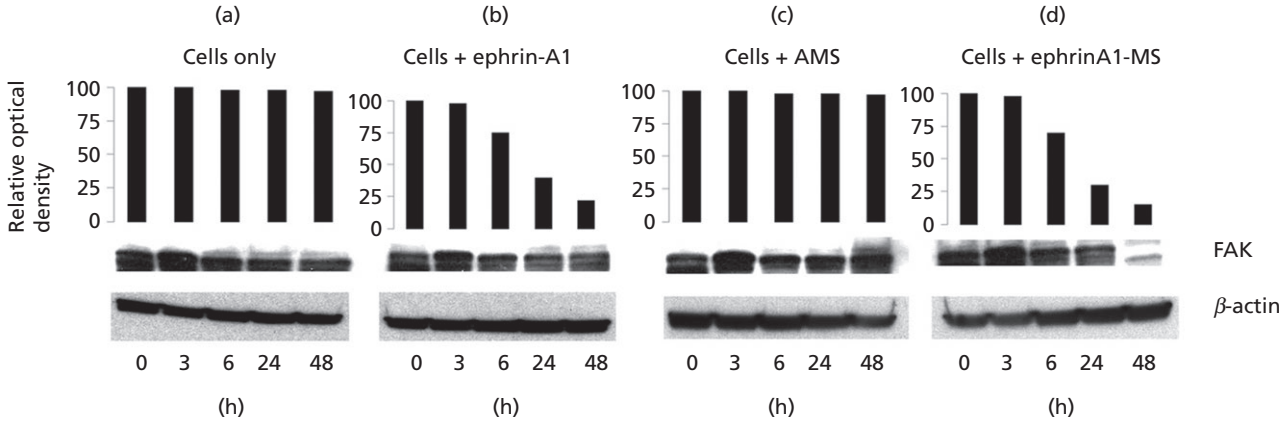

Figure 5 Effect of ephrinA1-loaded albumin microspheres on FAK expression in A549 cells. EphrinA1-loaded albumin microspheres (ephrinA1-MS) decreased focal adhesion kinase (FAK) expression in A549 cells within 48 h. The bars represent the densitometry values of the bands. The lower band, β-actin, was a housekeeping gene to show equal loading of protein. AMS, albumin microspheres.

Effect of ephrinA1-MS on FAK expression and migration of NSCLC cells

In the untreated A549 cells the expression of FAK remained constant, indicating that the cells grew and spread normally within 48 h as determined by Western blot analysis (Figure 5a). However, the cells treated with free ephrinA1 and ephrinA1-MS showed downregulation of FAK in Western blot analysis (Figure 5b). A significant decrease in FAK expression was noted after 6 h of incubation and then it decreased gradually up to 48 h. However, after 48 h, the FAK decrease was more prominent in ephrinA1-MS treated cultures compared with free ephrinA1 treated cultures. In addition, control AMS did not affect the expression of FAK (Figure 5c). This demonstrates that the ephrinA1-MS can effectively inhibit the spreading of A549 cells via downregulation of adhesion proteins (FAK).

Cell migration was measured by a wound healing assay. The A549 cells in control cultures almost closed up the wounded area in 18 h (Figure 6a). The cells treated with ephrinA1-MS showed a slower cell migration rate than untreated cells and cells treated with free ephrinA1. When the A549 cells were treated with higher concentrations of ephrinA1-MS, the boundaries of the cell monolayer showed a slower migration rate to heal the wound, which indicates that the efficacy of ephrinA1-MS can be enhanced by increasing the concentration (Figure 6b). The cell migration in control AMS treated cultures was unaffected and the cell migration

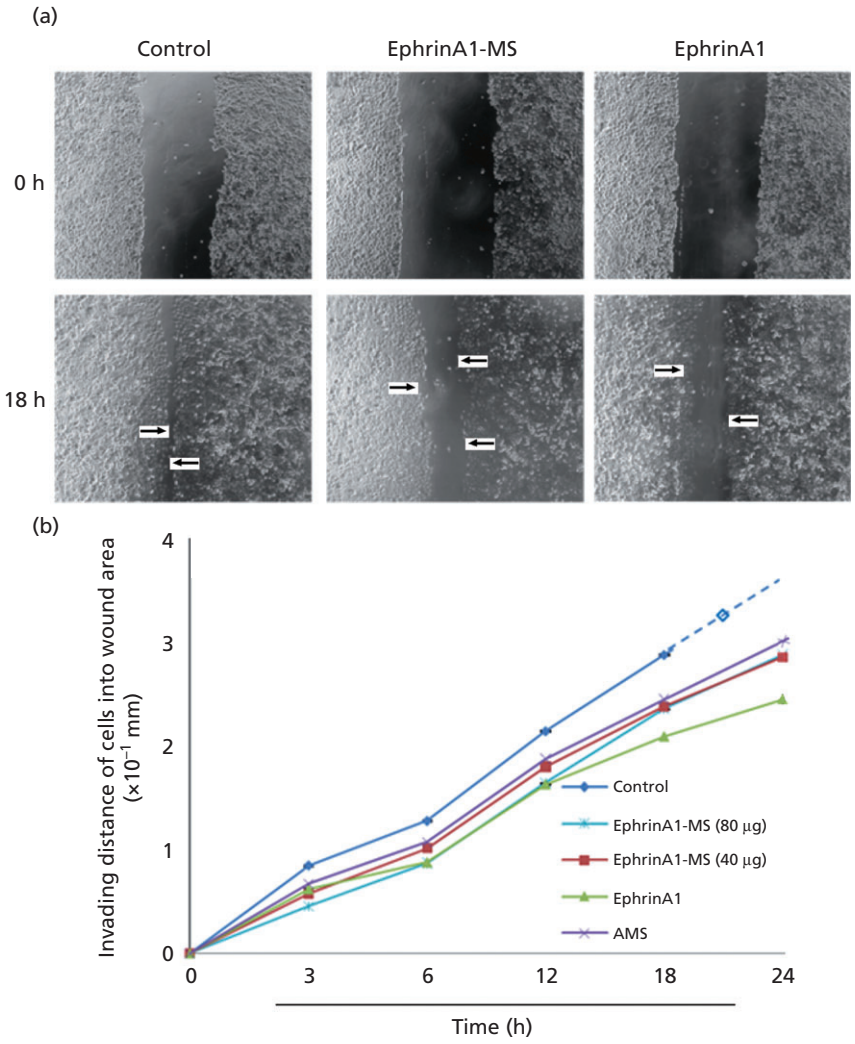

Figure 6 Effect of ephrinA1-loaded albumin microspheres on A549 cell migration. (a) Control cells migrated over the wounded area within 18 h, and the wound without treatment almost closed up. However, the wound treated with recombinant ephrinA1 or ephrinA1-loaded albumin microspheres (ephrinA1-MS) showed inhibition on wound recovery. Arrows indicate the migration of cells into the wounded area. (b) Wound healing rate with and without treatment. The wound treated with a higher dose of ephrinA1-MS showed enhanced inhibition on wound recovery. The data presented are the mean of three independent experiments. The control cells migrated and covered the whole wounded area within 24 h. The dashed line is the imagined extension of the solid line and the point on the dashed line shows the expected time that the wound would be healed.) AMS, albumin microspheres.

rate was comparable with untreated cultures. This indicates that the control AMS alone did not affect cell migration *in vitro*.

Effect of ephrinA1-MS on NSCLC tumour growth

A549 cells were seeded in cross-linked matrigels containing recombinant ephrinA1, AMS and ephrinA1-MS. The supernatant medium was replaced by fresh medium every 3 days. After 6 days of incubation, a high density of tumour colonies was observed in the matrigel containing media alone and control AMS. However, the density of tumour colonies in the matrigel containing free ephrinA1 and a high concentration of ephrinA1-MS was lower than the untreated cultures. When the concentration of ephrinA1-MS in the matrigel was higher than 20 μg/ml, the number of tumour colonies was significantly reduced, and a high concentration of ephrinA1-MS completely inhibited tumour formation (Figure 7a). In free ephrinA1 treated cultures the number as well as the size of tumour colonies was more obvious compared with ephrinA1-MS treated cultures. This may be due to constant release of ephrinA1 from ephrinA1-MS in the matrigel over time, whereas free ephrinA1 may denature over time. Consequently, after 12 days of incubation, the tumour growth was restored in free ephrinA1 treated cultures, whereas in ephrinA1-MS treated cultures the tumour growth was attenuated (Figure 7b). Increased cell spreading and tumour growth was found in untreated as well as free ephrinA1 treated cultures. These data suggest that synthesized ephrinA1-MS have the potential to effectively inhibit tumour growth compared with free ephrinA1 alone or control cells.

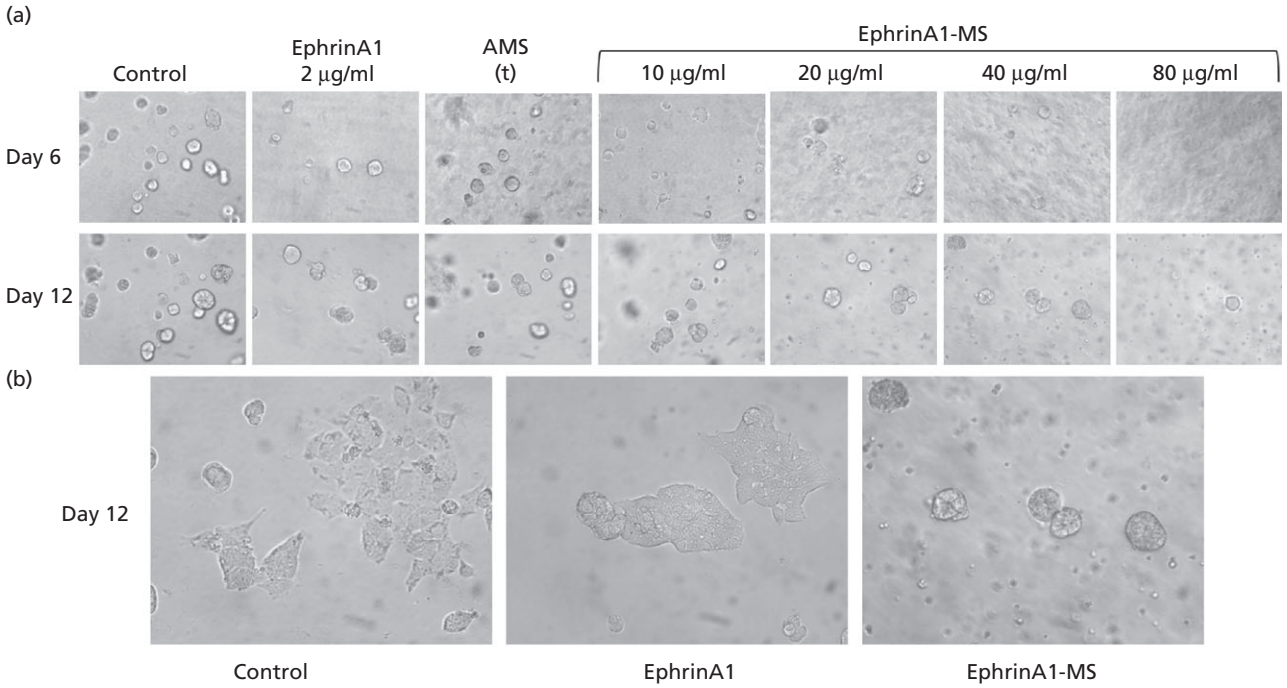

Figure 7 Effect of ephrinA1-loaded albumin microspheres on non-small cell lung cancer tumour growth. (a) Tumour growth decreased in A549 cells activated with ephrinA1-loaded albumin microspheres (ephrinA1-MS). The images were taken after 6 and 12 days of cultures in matrigel with serum-free medium. EphrinA1-MS and recombinant ephrinA1 showed smaller tumour colonies than untreated cells (control). (b) Spread of tumour cells. Untreated cells showed significant spreading on the matrigel after 12 days; the spread was inhibited in ephrinA1-MS treated cells. AMS, albumin microspheres.

Discussion

Receptor EphA2 expression is associated with poor prognosis. NSCLC cells are known to overexpress the receptor EphA2.[30] In earlier studies, we reported that activation of receptor EphA2 with its ligand ephrinA1 inhibits receptor EphA2 expression, leading to attenuation of tumour growth.[29] The present study is the first to show that AMS-conjugated ephrinA1 prolongs the antitumourogenic effect of recombinant ephrinA1 in NSCLC cells.

The water in organic solvent emulsion system provides a convenient method for the preparation of AMS. All the surfactants, emulsifiers and organic solvent in the emulsion system can be easily removed from the surface of AMS. The high surface hydrophilicity, high dispersity and high stability in aqueous solution of synthesized AMS allow the microspheres to be easily applied into biological systems. In this study, the AMS were mostly controlled over the size range of 5–10 μm to prevent fast protein release and diffusion. However, particle size is one of the most important factors in delivery efficacy, along with protein release rate and particle uptake efficiency by cells and tissues. When A549 cells were incubated with AMS, greater than 60% of cells had phagocytosed AMS in 40 min, and after 3 h of incubation the percentage of cells that phagocytosed AMS reached 90%. This indicates that the AMS can be efficiently phagocytosed by A549 cells over the particle size range of 5–10 μm. In addition, the cytotoxicity of AMS was very low or negligible when the concentration of AMS was below 0.5 mg/ml.

Proteins can be loaded in the matrix of microspheres by physical interaction between loaded proteins and the matrix, and can also be chemically conjugated on the surface of AMS through imine formation.[11,13] Protein loading efficiency in the matrix depends on the solubility of protein in the solution, swelling degree of AMS in solution and affinity interaction between the protein loaded and the matrix. However, the most important factor related to the loading efficiency on the surface is the density of aldehydes on the AMS surface because the aldehyde groups crosslink primarily with lysine in the proteins.[11] The proteins loaded in the matrix can be released quickly by diffusion, while those loaded on the surface will be released slowly through enzymatic lysis. As a result, the ephrinA1 loaded in AMS can bind EphA2 receptors through two possible approaches: by the ephrinA1 freely released into the media and by the ephrinA1 that stays conjugated with the AMS.

The ephrinA1-MS synthesized in this study contained about 10% of the total ephrinA1 loaded in the matrix and the rest of the protein was conjugated on the surface of the microspheres. The combination of matrix loading and surface conjugation of ephrinA1 in ephrinA1-MS can provide a higher effective concentration in a short time and a prolonged continuous release from the surface over a longer period. Due to the high phagocytic rate of ephrinA1-MS, the release of ephrinA1 takes place inside and outside the cell membrane at the same time. This differs from the general delivery method which can only diffuse free in the environment outside the cells. This delivery method may improve the efficiency for

lung cancer treatment by targeting EphA2. In addition, this system could also be used for a variety of other target proteins in other cancers.

EphrinA1-MS can effectively inhibit cell spreading and migration of A549 cells *in vitro*. When added to the wells plated with A549 cells, the ephrinA1-MS precipitated on the bottom of wells within minutes and stayed on the monolayer of cells, which implies that the ephrinA1-MS can release ephrinA1 directly on top of cell monolayers and provide a higher drug concentration over time in the milieu of target cells. In the present study, using ephrinA1-MS we were able to deliver the ephrinA1 directly onto the target cells and this was effective in inhibiting tumour cell migration. Free ephrinA1 can only be added to media-containing wells and it may distribute evenly in culture media and be less effective. The microspheres showed good retention on the cell monolayer: the injected ephrinA1-MS dispersed in the matrigels and provided a localized high density of microspheres without ephrinA1 being diffused. Compared with ephrinA1-MS, although initially the free ephrinA1 added to the system provided a faster and higher concentration binding to cells, the free ephrinA1 is eventually diffused into the matrigels and thus over time it was less effective at inhibiting tumour growth.

In the cell migration study, when the wounded culture was treated with ephrinA1-MS these cultures showed a slower healing rate and the inhibition of cell migration was enhanced with increasing concentration of ephrinA1-MS. Similar results were also noticed in the 3D tumour assay. The cell spreading was significant around the surface area of the matrigel containing free ephrinA1 because the free ephrinA1 in the matrigel may have degraded over time. However, cell spreading was not found on the surface of matrigel containing ephrinA1-MS due to the higher retention and constant release of ephrinA1 over time from the microspheres. The AMS synthesized here may serve as potential protein carriers for different delivery purposes. The high dispersity and stability in aqueous solution enables the ephrinA1-MS to be applied in endobronchial intratumoural chemotherapy and intratumoural therapies for other cancers.

Conclusions

This study demonstrates that ephrinA1 conjugated on the surface of AMS remained stable and efficient to inhibit NSCLC cell growth and migration after being released locally from the microspheres via phagocytosis and degradation. Moreover, the low cytotoxicity and high phagocytic rate allow the AMS to be applied to various other delivery systems. The present data provide a promising foundation for future in-vivo studies and offer an exciting avenue for the development of receptor-targeted therapeutic drugs that may help in the management of patients with NSCLC.

Declarations

Conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

Funding

This work was financially supported by a Florida Department of Health grant award (09KW-08).

Acknowledgement

Technical assistance by Zita Burkhalter is acknowledged.

References

1. CDC. Work-Related Lung Disease Surveillance Report. NIOSH Publication, 2002: Atlanta, GA: CDC. http://www.cdc.gov/niosh/docs/2003-111/2003-111.html
2. Goldberg EP *et al*. Intratumoral cancer chemotherapy and immunotherapy: opportunities for nonsystemic preoperative drug delivery. *J Pharm Pharmacol* 2002; 54: 159–180.
3. Celikoglu SI *et al*. Endobronchial intratumoral chemotherapy (EITC) followed by surgery in early non-small cell lung cancer with polypoid growth, causing erroneous impression of advanced disease. *Lung Cancer* 2006; 54: 339–346.
4. Celikoglu F *et al*. Bronchoscopic intratumoral chemotherapy of lung cancer. *Lung Cancer* 2008; 61: 1–12.
5. Celikoglu F *et al*. Intratumoural chemotherapy of lung cancer for diagnosis and treatment of draining lymph node metastasis. *J Pharm Pharmacol* 2010; 62: 287–293.
6. Cheung RY *et al*. In vivo efficacy and toxicity of intratumorally delivered mitomycin c and its combination with doxorubicin using microsphere formulations. *Anticancer Drugs* 2005; 16: 423–433.
7. Lammers T *et al*. Effect of intratumoral injection on the biodistribution and the therapeutic potential of HPMA copolymer-based drug delivery systems. *Neoplasia* 2006; 8: 788–795.
8. Burgess DJ, Hickey AJ. Microspheres technology and applications. In: Swarbric JB, Boylan JC, eds. *Encyclopedia of Pharmaceutical Technology*. New York: Marcel Dekker, 1995: 1–29.
9. Okada H, Toguchi H. Biodegradable microspheres in drug-delivery. *Crit Rev Ther Drug Carrier Syst* 1995; 12: 1–99.
10. Kawaguchi H. Functional polymer microspheres. *Prog Polym Sci* 2000; 25: 1171–1210.
11. Longo WE *et al*. Preparation of hydrophilic albumin microspheres using polymeric dispersing agents. *J Pharm Sci* 1982; 71: 1323–1328.
12. Longo WE, Goldberg EP. Hydrophilic albumin microspheres. 1985; 18–26.
13. Jones PDE, Illum L. Attachment of monoclonal antobodies to microspheres. 1985; 67–84.
14. Almond BA *et al*. Efficacy of mitoxantrone-loaded albumin microspheres for intratumoral chemotherapy of breast cancer. *J Control Release* 2003; 91: 147–155.
15. Cohen S *et al*. Controlled delivery systems for proteins based on poly(lactic glycolic acid) microspheres. *Pharm Res* 1991; 8: 713–720.
16. Blanco D, Alonso MJ. Protein encapsulation and release from poly(lactide-co-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants. *Eur J Pharm Biopharm* 1998; 45: 285–294.
17. Grenha A *et al*. Microencapsulated chitosan nanoparticles for lung protein delivery. *Eur J Pharm Sci* 2005; 25: 427–437.
18. Bouillot P *et al*. Protein encapsulation in biodegradable amphiphilic microspheres. *Int J Pharm* 1999; 181: 159–172.
19. Stivaktakis N *et al*. Immune responses in mice of beta-galactosiclase adsorbed or encapsulated in poly(lactic acid) and poly(lactic-co-glycolic acid) microspheres. *J Biomed Mater Res A* 2005; 73A: 332–338.

20. Nam YS, Park TG. Protein loaded biodegradable microspheres based on PLGA-protein bioconjugates. *J Microencapsul* 1999; 16: 625–637.
21. Takeoka S *et al*. Conjugation of von Willebrand factor-binding domain of platelet glycoprotein Ib alpha to size-controlled albumin microspheres. *Biomacromolecules* 2000; 290–295.
22. Berner VK *et al*. Conjugation of protein antigen to micro-particulate beta-glucan from *Saccharomyces cerevisiae*: a new adjuvant for intradermal and oral immunizations. *Appl Microbiol Biotechnol* 2008; 80: 1053–1061.
23. Keegan ME *et al*. In vitro evaluation of biodegradable microspheres with surface-bound ligands. *J Control Release* 2006; 110: 574–580.
24. van de Weert M *et al*. Protein instability in poly(lactic-co-glycolic acid) microparticles. *Pharm Res* 2000; 17: 1159–1167.
25. Walker-Daniels J *et al*. Overexpression of the EphA2 tyrosine kinase in prostate cancer. *Prostate* 1999; 41: 275–280.
26. Ogawa K *et al*. The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. *Oncogene* 2000; 19: 6043–6052.
27. Carter N *et al*. EphrinA1-induced cytoskeletal re-organization requires FAK and p130(cas). *Nat Cell Biol* 2002; 4: 565–573.
28. Brannan JM *et al*. EphA2 in the early pathogenesis and progression of non-small cell lung cancer. *Cancer Prev Res (Phila)* 2009; 2: 1039–1049.
29. Nasreen N *et al*. Receptor EphA2 activation with ephrinA1 supresses growth of malignant mesothelioma (MM). *Cancer Lett* 2007; 258: 215–222.
30. Brannan JM *et al*. Expression of the receptor tyrosine kinase EphA2 is increased in smokers and predicts poor survival in non-small cell lung cancer. *Clin Cancer Res* 2009; 15: 4423–4430.