Cancer Chemother Pharmacol (2008) 61:997–1005
DOI 10.1007/s00280-007-0557-x

ORIGINAL ARTICLE

# The preparation and characterization of anti-VEGFR2 conjugated, paclitaxel-loaded PLLA or PLGA microspheres for the systemic targeting of human prostate tumors

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Received: 17 November 2006 / Accepted: 28 June 2007 / Published online: 15 August 2007


**Abstract**
*Purpose* The objective of this study was to manufacture paclitaxel (PTX) loaded polymeric microspheres, that were surface conjugated with antibodies to vascular endothelial growth factor receptor 2 (anti-VEGFR2), for systemic targeting to angiogenic sites in prostate tumors.
*Methods* Microspheres were manufactured in the 1–3 μm size range from poly (l-lactic acid) (PLLA) or poly (lactide-co-glycolide) (PLGA) by a modified solvent evaporation method using Polytron homogenization followed by high speed dispersion in poly vinyl alcohol. Antibodies were conjugated to the surface of these microspheres using cyanogen bromide activation of the polymer surface. Cell Binding was determined using human umbilical vein endothelial cells (HUVECs) in vitro. Efficacy determinations were made using human prostate tumors (PC-3) grown subcutaneously in mice.
*Results* Antibodies were effectively bound to the surface of PLLA and PLGA micropsheres. Anti-VEGFR2 conjugated PLLA microspheres bound strongly to HUVEC's. Pilot efficacy studies in mice showed variability but demonstrated a significant inhibition of tumor growth following the systemic administration of a single dose of PTX-loaded anti-VEGFR2 conjugated PLLA microspheres as compared to non-antibody-conjugated PTX-loaded microspheres.
*Conclusion* Anti-VEGFR2 conjugated PLLA microspheres containing PTX may offer an effective way of administering a controlled release formulation of the drug to target prostate tumors.

**Keywords** Paclitaxel · Microspheres · Anti-VEGFR · Prostate cancer

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## Introduction

Currently the anticancer drug paclitaxel (PTX) is administered intravenously in a Cremophor EL®/alcohol micellar formulation (Taxol®) and is associated with serious toxic side effects in patients [28]. Taxol administration is also associated with high initial plasma drug concentrations followed by rapid drug clearance [28]. There is currently no intravenous formulation of PTX that avoids the toxic side effects associated with the Cremophor-EL vehicle and the high peak plasma drug levels, or provides sustained therapeutic levels of the drug. Controlled release, submicron sized PTX-loaded systems such as micelles, nanoparticles or liposomes effectively solubilize PTX but these systems do not ensure longer drug circulation times and there are limited reports of the effective therapeutic use of these systems [9, 25, 29, 31].

Paclitaxel has been encapsulated at high efficiency in polymeric microspheres manufactured from polyesters such as poly (lactic acid), poly (caprolactone) and poly (lactide-co-glycolide) (PLGA) [7, 12, 22]. These microspheres were manufactured using a solvent evaporation method that allows for high yields and high levels of drug encapsulation (greater than 20% PTX loading). These formulations have

been shown to release PTX in a controlled manner over many weeks and to provide anticancer [22], antiangiogenic [7] and antiarthritic [23] effects following localized administration. It is now recognized that microspheres in the 1–3 μm size range may allow for unrestricted circulation of microspheres [1, 6] without physical entrapment by capillaries. However, the effective manufacture of microspheres in such a narrow and small size range is extremely difficult using established methods [10]. Furthermore, polymeric microspheres may be opsonized in plasma by IgG or complement and rapidly cleared from the blood by the reticuloendothelial system [1, 17]. Since PTX releases very slowly, over a period of months, from polymeric microspheres [7, 22], most of the encapsulated drug would be unlikely to be released.

A PTX microspheres formulation specifically targeted to tumor sites, where the drug payload was released in a controlled manner at effective levels, would represent a significant advancement in cancer chemotherapy. The use of microspheres with surface conjugated antibodies to targets on cancer cells has been shown to allow for effective binding of microspheres in vitro [6]. However, in vivo, systemically administered microspheres are prevented from access to tumor cell binding sites by capillary walls [1, 6] so there are few reports describing the effective use of antibody-conjugated drug loaded microspheres in vivo [33]. Although tumor cells may not offer an attractive target for systemically delivered microspheres, angiogenic sites within tumors are composed of proliferating capillary cells which are fully exposed and directly available to the binding ligand [5, 26].

In this work, the objective was to deliver small micron-sized PTX-loaded microspheres to target angiogenic sites on capillaries leading to tumors with potential application in prostate cancer. PTX-loaded polymeric pastes that may be injected locally and provide a controlled release of the drug in a controlled manner have been previously shown to strongly inhibit human prostate tumor growth in a xenograft mouse model [16]. PTX is particularly suited to local delivery within the capillary cells of tumors since it is known to inhibit both tumor cell proliferation and tumor angiogenesis, hence providing activity against dual aspects of the disease [7, 22].

The approach of this study was to bind vascular endothelial growth factor receptor antibodies (anti-VEGFR2) to the surface of PTX-loaded microspheres prepared in the 1–3 μm size range. Because very low molecular weight (2,000 g/mol) poly (l-lactic acid) (PLLA) microspheres degrade and release PTX rapidly compared to much higher molecular weight PLGA microspheres [12, 22] both polymers were used in these studies since PTX release rate would be a significant factor in effective tumor regression.

Both the VEGFR1 (Flt-1) and VEGFR2 (Flk-1) receptors are selectively expressed on endothelial cells and are highly upregulated in proliferating (angiogenic) capillary cells of numerous types of prostate tumors [2, 20]. Prostate cancer cells are known to produce VEGF to stimulate tumor angiogenesis and there are numerous reports describing increased expression of VEGF in cancer cells from localized and metastatic tumors as well as in benign prostatic hyperplasia tissues [2, 15, 34]. VEGFR2 has become the most attractive endothelial target in all cancers because of its known control of angiogenesis [34]. Because the VEGF receptor-2 is highly expressed in proliferating (not quiescent) capillary cells [2, 20], the angiogenic beds of tumors may provide microsphere binding sites to retain the microspheres at the required site of action. In this work, effective binding of anti-VEGFR2 conjugated PLLA microspheres to human umbilical vein endothelial cells (HUVECs) grown in cell culture was established. This was followed by pilot studies using similar, anti-VEGFR2, PTX-loaded, microspheres in mice bearing subcutaneous human prostate tumors (PC-3) with analysis of tumor location using whole animal imaging and tumor size by caliper measurement over 3 weeks.

## Materials and methods

### Materials

Poly (l-lactic acid) (molecular weight 2K g/mol) was obtained from Polysciences Inc. (Warrington, PA, USA). Poly (lactic-co-glycolic acid) (85/15 ratio, IV = 0.61) was obtained from Birmingham Polymers Inc. (Birmingham, AL, USA). PTX was obtained from PolymedT Inc. (Houston, TX, USA). Poly vinyl alcohol (PVA) (98% hydrolyzed, molecular weight 13–23 KDa) was obtained from Aldrich (Oakville, ON, Canada). Cyanogen bromide and fluorescein isothiocyanate conjugated IgG (FITC-IgG) was obtained from Sigma Chemical Co (St Louis, MO, USA). The VEGFR2 antibody (anti-VEGFR2: binds to mouse and human receptors) was purchased from Upstate Inc (Charlottesville, VA, USA). All solvents were HPLC grade and purchased from Fisher Scientific (Ottawa, ON, Canada). HUVECs were obtained from Biowhittikar/Cambrex (Walkersville, MD, USA) and cultured in specific media from this company.

### Methods

#### *Preparation of paclitaxel loaded microspheres*

Microspheres were manufactured using a modified solvent evaporation method. Polymer and PTX were dissolved at a

final polymer concentration of 5% w/v in dichloromethane (DCM) in 4 ml of solution. A 10% (w/v) stock solution of PVA in water was made by stirring the solution at 95°C for 30 min. This solution was then stored at 4°C and diluted in water to the final concentration in a 100 ml volume used for microsphere manufacture. Eighty milliliters of PVA solution was placed in a 500 ml plastic container (vertical sides) and stirred at 1,500 rpm using an overhead stirrer. The remaining 20 ml of PVA was placed in a 50 ml plastic centrifuge tube and dispersed at high speed (17,000–29,000 rpm) using a Polytron homogenizer. The polymer/drug solution was pipetted into the 20 ml of PVA over a period of 1 min and dispersion was continued for 3 min. This PVA/microsphere suspension was then quickly poured into the remaining 80 ml of PVA solution stirring at 1,600 rpm. The system was then left stirring for 2 h, the microspheres were washed four times in water by centrifugation at 1,500×*g* for 5 min and dried under vacuum for 3 days.

The amount of drug encapsulated in the microspheres was measured as previously described [22]. Briefly 5 mg of microspheres were dissolved in 1 ml of DCM and then 15 ml of acetonitrile to water [60:40 ratio (v/v)] were added with vigorous shaking. The contents were allowed to settle and the amount of PTX in the top and bottom phases were determined by HPLC using a Waters Millennium system that utilized a mobile phase of 57% acetonitrile, 5% methanol, 38% water, flowing at 1 ml/min, a C18 reverse phase Novapak column (Waters), a 20 µl injection volume, with detection at 232 nm.

*Microsphere size, morphology and yield determinations*

The size distribution of microspheres was determined using a Malvern Mastersizer 2000 (Malvern Inc., Malvern, Worcestershire, UK). The morphology of microspheres was determined using scanning electron microscopy using a Hitachi S-300N system following gold-palladium alloy Sputter coating of the microspheres. The yield was determined by weighing microspheres after drying.

*Conjugation of FITC-IgG or anti VEGFR2 antibodies to microspheres*

The conjugation of antibodies to microspheres was accomplished using a cyanogen bromide activation method as previously described [13]. The microspheres were suspended in dilute NaOH (pH 11) at 100 mg/ml for 5 min and then washed and treated with cyanogen bromide (1% w/v in dilute NaOH, pH 11) for 10 min followed by three washes in water and two washes in 0.1 M sodium bicarbonate solution. The microspheres were then suspended at 5 mg/ml in the FITC-IgG in a volume of 1 ml (various FITC-IgG concentrations) or 100 mg/ml in the anti-VEGFR2 (400 µg/ml) solution in a volume of 10 ml and tumbled, overnight in a sealed darkened tube with rotation at 10 rpm. The microspheres were then washed in water once and twice in 0.1 M sodium bicarbonate solution and were used within 24 h.

The extent of FITC-IgG conjugation to microspheres was determined as follows. Five milligrams of microspheres were suspended in 1 ml of 1% w/v trypsin in 0.1 M sodium bicarbonate solution and incubated at 37°C for 2 h with tumbling at 40 rpm. The tubes were centrifuged at 14,000×*g* for 30 s, and the supernatants were collected and the amount of FITC-IgG was then determined using quantitative fluorescence spectroscopy. Fluorescence was measured on a Cary Eclipse spectrofluorometer with an excitation wavelength of 488 nm and an emission wavelength of 517 nm.

*Binding of anti-VEGFR2 conjugated PLLA microspheres to HUVEC cells in vitro*

Human umbilical vein endothelial cells were grown in culture on 48-well plates. For binding experiments, cells were seeded at 15,000 cells per well and left to equilibrate for 3 days. Either control or anti-VEGFR2 antibody-conjugated PTX-loaded (20% w/w) PLLA microspheres were then suspended at 2.5 mg/ml in HUVEC media and 300 µl of the suspension (0.75 mg of microspheres) were added to the cells. The microspheres were then left to sediment under gravity on top of the cells to allow antibody-receptor binding to occur over a 3-h period. After 3 h, each well was gently washed (six washes) with 1 ml of media followed by aspiration to remove unbound microspheres. The extent of microspheres binding to the cells was then determined either qualitatively using optical imaging (Motic AE31optical microscopy with an infinity3 digital imaging attachment (VWR scientific, Toronto, ON, Canada) or quantitatively, by dissolution of the entire contents of the wells in DMSO followed by extraction/quantitation of PTX by HPLC as described above.

*In vitro release of paclitaxel from microspheres*

Ten milligrams of 20% PTX-loaded PLLA or PLGA microspheres were placed in 16 ml screw cap (Teflon) glass tubes and 15 ml of phosphate buffered saline (10 mM PBS, pH 7.4) were added. The tubes were rotated end-over-end at 8 rpm at 37°C. At appropriate times, the tubes were centrifuged at 1,000×*g* for 5 min, the PBS was removed and saved and fresh PBS was placed in the tube for re-incubation. One milliliter of DCM was added to the PBS samples and the contents were shaken in a capped tube for 30 s. The contents were then allowed to phase separate for 30 min and the aqueous phase (drug-free) was aspirated from the

1 ml DCM phase (drug-rich). The DCM was then evaporated to dryness at 40°C under nitrogen gas and the contents were re-dissolved in 1 ml of acetonitrile:water (60:40 v/v) and analyzed by HPLC methods described above.

*Efficacy of anti-VEGFR2 conjugated, paclitaxel-loaded microspheres*

All animal studies were carried out in accordance with the UBC Animal Care Committee guidelines. The PC-3 tumor model was used as previously described [32]. Briefly, human PC-3 prostate tumors were grown in mice (Balb/c strain mice 6–8 weeks old and weighing approximately 25 g, obtained from Charles River Laboratory, Montreal, QC, Canada). Following 2 weeks equilibration, tumor cells were injected subcutaneously as previously described [32] and the mice were left untreated until the tumors were palpable masses (approximately 300 $mm^3$). Human prostate tumor (PC-3 tumors at 300 $mm^3$)-bearing mice were randomly assigned to two groups and treated by tail vein injection with 20 mg of PTX-loaded PLLA microspheres (20% drug loading) (PTX dose of 4 mg) which were either untreated (non-conjugated) or anti-VEGFR2 conjugated PLLA microspheres ($n = 12$/group). A smaller secondary control group of five mice were injected with 100 µl of saline. The mice were then left untreated and the tumor volume was measured weekly for a month. Due to the large variation in original tumor sizes between mice, the tumor growth was then calculated as a % of original size and expressed as a fold increase in tumor size.

## Results

Preparation and characterization of microspheres in the 1–3 µm diameter range

In initial optimization studies, microspheres were manufactured without the Polytron homogenizer step. Rather, the PLGA or PLLA solution was pipetted directly into the 100 ml of 1% (w/v) PVA solution with overhead stirring at 4,000 rpm. At room temperature, this method produced microspheres with a mean diameter of 5 µm and a range from 1 to 20 µm. By cooling the PVA and polymer solution to 4°C, both the mean diameter and range were reduced to 4 µm and 1 to 13 µm, respectively. However, when the Polytron homogenization step was included (27,000 rpm) at a temperature of 4°C, the mean diameter dropped to 2.5 µm and a range of 1–7 µm. As the dispersion stir speed increased from 15,000 to 27,000 rpm, there was a decrease in the mean diameter of the microspheres from 4 to 2 µm.

Using a dispersion speed of 23,000 rpm for 5 min and then an overhead stirrer at 1,600 rpm for 2 h, the mean size of microspheres could also be partially controlled by varying the concentration of PVA. Using PVA concentrations of 5, 2.5 and 1% the mean diameters of the PLGA microspheres were 0.98 to 1.2 µm and 1.4 µm, respectively.

Using the same stirring rates (23,000 rpm for 5 min and 1,600 rpm for 2 h) the mean size of the PLGA microspheres also depended on the PLGA concentration in the DCM, dropping from 5 to 1.4 µm as the PLGA concentration (in DCM) decreased from 10% (w/v) to 1% (Fig. 1).

Following these experiments, microspheres were manufactured at room temperature using a 2.5% polymer solution, a 1% PVA concentration and a polytron stir speed of 23,000 rpm for 5 min followed by overhead stirring for 2 h. For both PLLA and PLGA, these methods produced microspheres with a mean diameter of 1.4 µm and a range of 1–5 µm (Fig. 1).

We report the use of PVA supplied by Aldrich with a molecular weight of approx 13,000–23,000 g/mol and degree of acetylation of 98% for the solvent evaporation manufacturing method. It was observed that although many sources of PVA could be used to manufacture PLGA or PLLA microspheres in the appropriate size range, only this source of PVA allowed for high yields and effective PTX encapsulation. Frequently PTX was observed to crystallize out of the microspheres as long thin needles when other sources of PVA were used. The reason for this finding is not known. However, we speculate that since the degree of acetylation of the PVA may affect the thickness of the multimolecular layer film of PVA at the forming microsphere surface, it is possible that the rate of loss of DCM from the microspheres may have varied with different PVA solutions, affecting the nature of PTX precipitation in the microspheres.

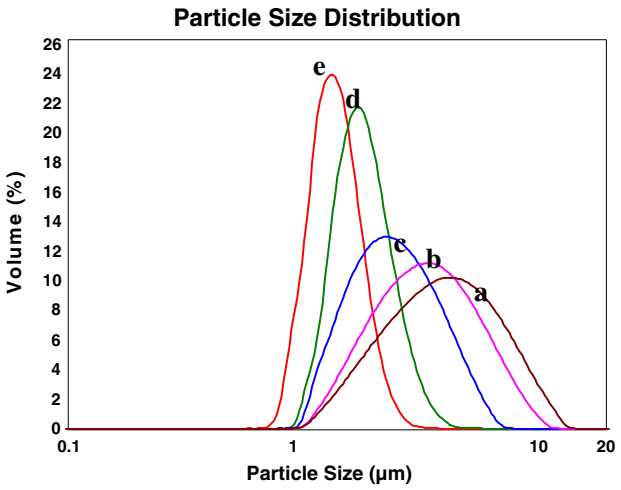


**Fig. 1** Effect of PLGA concentration on the particle size distribution of PLGA microspheres. PLGA microspheres manufactured at 4°C using polytron homogenization at 23,000 rpm for 5 min then overhead stirrer at 1,600 rpm for 2 h in a PVA concentration of 1% and a PLGA concentration of *e*: 1%, *d*: 2.5%, *c*: 5%, *b*: 7.5% and *a*: 10% w/v

## Microsphere characterization: morphology and drug loading

Poly (lactide-co-glycolide) microspheres were uniformly shaped with a smooth surface whereas PLLA microspheres were smooth but non-uniform and dimpled as seen in Fig. 2. The drug PTX encapsulated at greater than 85% efficiency in both PLGA and PLLA microspheres with microsphere yields always greater than 53%. Both yields and drug encapsulation efficiencies were slightly higher for PLGA compared to PLLA, but both polymers allowed for the effective manufacture of PTX-loaded microspheres in the appropriate size ranges (1–5 μm).

## Conjugation of antibodies to microspheres

Fluorescein isothiocyanate conjugated IgG was used as a probe antibody to quantitate the conjugation of antibodies to microspheres. Using the cyanogen bromide surface activation technique, FITC-IgG antibodies conjugated well to the surface of PLGA microspheres as shown in Fig. 3. The use of higher antibody concentrations and longer conjugation times increased the conjugation efficiency (Figs. 3, 4). Generally, the antibodies conjugated to PLGA and a 50:50 blend of the two polymers, PLGA and PLLA at higher efficiency than to PLLA microspheres at all antibody concentrations (Fig. 4). There was a general increase in the amount of FITC-IgG conjugated to the microspheres when the concentration was changed from 2 μg (antibody)/mg(microspheres) to 20 μg/mg. In studies using anti-VEGFR2 antibodies, a concentration of 4 μg/mg was used to ensure reasonable levels of coverage of the microsphere surface with conjugated antibodies.

## Binding of anti-VEGFR2 antibody-labeled PLLA microspheres to HUVECs

Anti-VEGFR2 antibodies were conjugated on PLLA microspheres using a starting concentration of 50 μg/ml in a volume of 10 ml containing 100 mg/ml microspheres and a 24 h incubation.

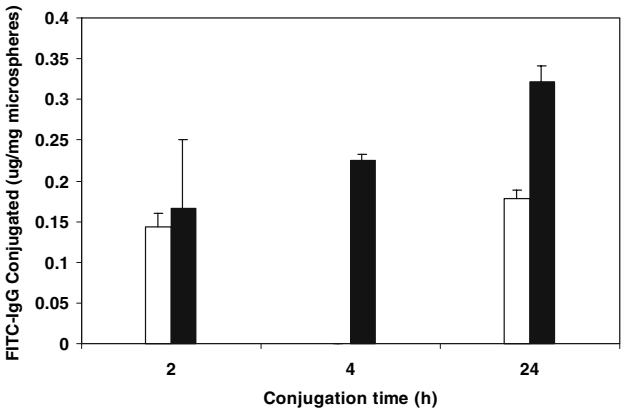


**Fig. 3** Effect of conjugation time and FITC-IgG concentration on the conjugation of FITC-IgG to PLGA microspheres (5 mg of 2.5 μm mean diameter) using cyanogen bromide surface activation methods. FITC-IgG concentrations: *open bar*: 2 μg/mg, *dark filled bar*: 10 μg/mg

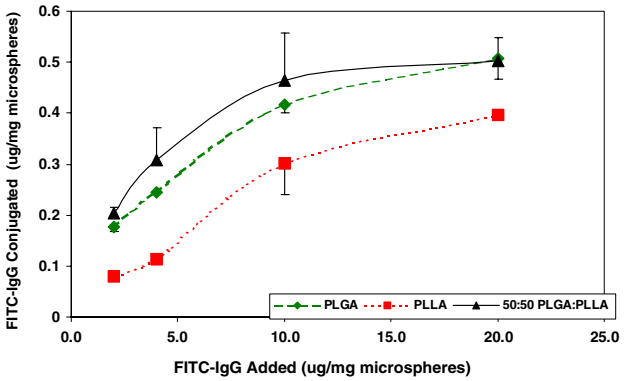


**Fig. 4** Effect of FITC-IgG concentration and microsphere composition on the conjugation of FITC-IgG to *PLGA*, *PLLA* or microspheres manufactured from a 50:50 w:w blend of *PLGA:PLLA*. (5 mg of microspheres with mean diameters of 2.5 μm using cyanogen bromide conjugation over 24 h)

These microspheres were found to bind well to HUVECs as shown in Fig. 5a, b. In general, these antibody-conjugated microspheres bound to PLLA microspheres approximately 50 times more efficiently than unconjugated control microspheres and longer incubation times allowed for increased cell binding (Fig. 5a). The effective binding of

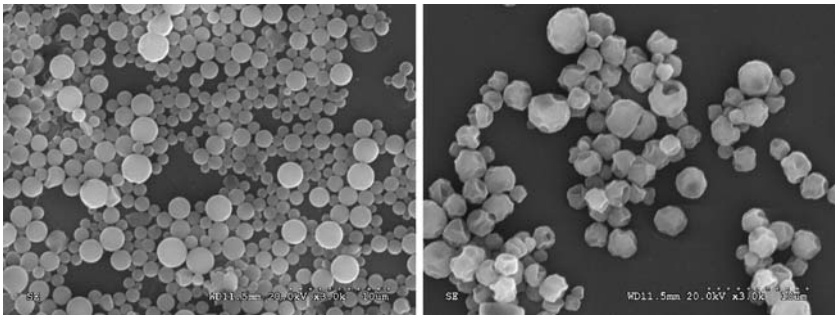

**Fig. 2** Microsphere morphology: scanning electron micrographs of paclitaxel loaded (20% w/w) PLGA (*left*) or PLLA (*right*) microspheres, manufactured using 1% PVA, a polytron speed of 23,000 rpm followed by overhead stirring at 1,600 rpm

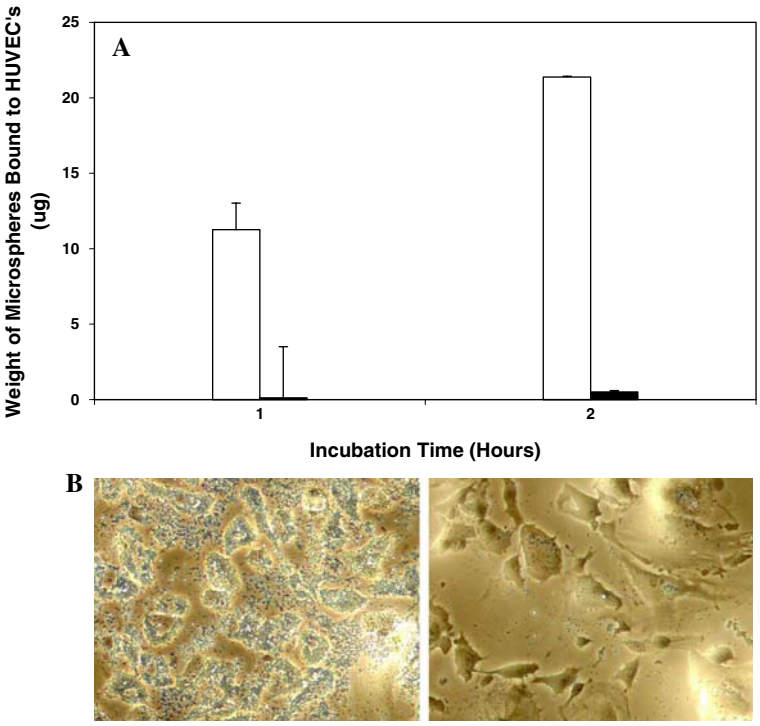


**Fig. 5** **a** Effect of incubation time on the binding of paclitaxel-loaded (20%) anti-VEGFR2 antibody-conjugated PLLA microspheres (2.5 μm diameter) to HUVECS. [0.75 mg of control (*dark filled bar*) or antibody-conjugated microspheres (*open bar*) incubated with HUVECS at 10,000 cells per well in a 48 well plate.] **b** Optical microscopy of HUVEC's treated with (*left photograph*): anti-VEGFR2 antibody-conjugated PLLA microspheres (2.5 μm diameter) or (*right photograph*): control PLLA microspheres, washed six times to remove unbound microspheres. (microspheres may be observed as *small dark dots*)

anti-VEGFR2 conjugated PLLA microspheres (seen as small dark dots) to HUVECs can be clearly observed in Fig. 5b.

## Paclitaxel release from microspheres

Paclitaxel released from both PLGA and PLLA microspheres with a burst phase of drug release followed by a slower more sustained release over 2 weeks as shown in Fig. 6. The release from PLLA was more rapid than from PLGA microspheres so that at 14 days, 27% of the encapsulated drug had released from PLLA whereas only 8% had released from PLGA microspheres.

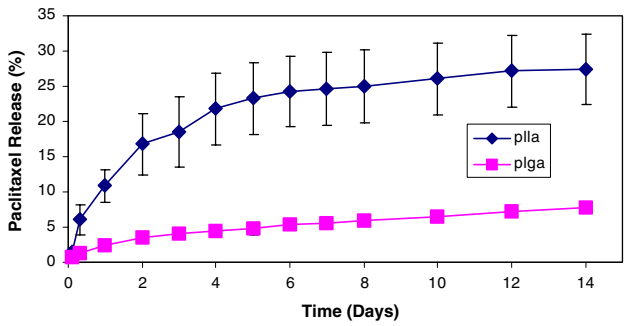


**Fig. 6** Release of paclitaxel (20% w/w) from *PLLA* (dark filled diamond) or *PLGA* (dark filled square) microspheres

## Pilot efficacy studies in mice bearing PC-3 tumors

Human PC-3 tumors grown subcutaneously in mice grew rapidly as shown in Fig. 7. When mice bearing these tumors were injected by tail vein injection with PTX-loaded (20% w/w loading), anti-VEGFR2 conjugated PLLA microspheres, an inhibition of tumor growth rate was observed over mice treated with unconjugated, PTX-loaded, microspheres (Fig. 7). The statistically significant increase in tumor volume in mice treated with unconjugated, PTX-loaded microspheres over those treated with anti-VEGFR2 conjugated, PTX-loaded microspheres is shown by asterisk (*) in Fig. 7. Using Student's *t*-test (2-tail analysis) the significance between tumor growth in animals treated with PTX-loaded, unconjugated microspheres compared to PTX-loaded anti-VEGFR2 conjugated microspheres was demonstrated with *P*-values of 0.008 and 0.017 at 2 and 3 weeks, respectively. This inhibition was significant at these time points but the experiment was terminated when the tumors in some mice became large and growth rates became erratic.

## Discussion

The optimal characteristics of a targeted microsphere formulation of PTX to treat prostate cancer are effective binding to target cells in the tumor, a high drug payload

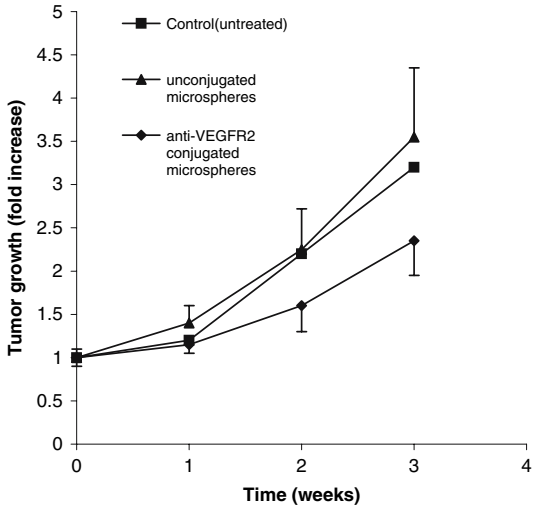


**Fig. 7** Effect of paclitaxel-loaded (20% w/w), anti-VEGFR2-conjugated PLLA microspheres (2.5 μm diameter) on the growth of human prostate tumors (PC-3) grown subcutaneously in mice. Following castration, mice were equilibrated until tumors had reached 300 $mm^3$ in volume and then injected with 20 mg of 20% paclitaxel-loaded control (no antibody) or anti-VEGFR2 conjugated microspheres in 300 μl of saline. Tumor volume was then measured at specified time points ($n$ = 12/group). *Asterisk* indicates significant difference between anti-VEGFR2 conjugated microsphere and control microsphere treatment groups at $P < 0.05$ using Student's $t$-test

(i.e., high levels of drug encapsulation) and a microsphere size range of approximately 1–3 μm. This size range is critical as it allows for free circulation and the potential to bind to angiogenic sites following multiple passes of the tumor site [1, 6].

Although the use of a solvent evaporation method of microsphere manufacture has been shown to allow for high PTX encapsulation efficiencies [7, 22, 23], the method is generally not suitable for the manufacture of microspheres below 10 μm in diameter. Using a solvent evaporation method with homogenization, Gupte and Ciftci [12], was able to manufacture PLGA microspheres with a mean diameter of 2.5 μm but the diameter ranged from sub micron to 18 μm and PTX was encapsulated at a loading of only 1.5%. There are numerous reports of alternative methods to manufacture small diameter polymeric microspheres, including extrusion, dripping, static mixing [10], spray drying [4] acoustic excitation [3] and the use of microporous glass membranes [27]. However none of these reports described the production of microspheres in an appropriate size range or containing PTX. In this study the classical solvent evaporation method has been greatly modified to allow for both high levels of PTX encapsulation in microspheres of the appropriate size range (1–5 μm diameter). The use of a Polytron homogenizer in combination with low PVA concentrations allowed for high shear rates and small but uniform microsphere size ranges (Fig. 1). The mean diameter of the microspheres was inversely proportional to both the stir speed and the PVA concentration and directly proportional to the polymer (PLGA) concentration in the dichloromethane. These relationships are in agreement with those previously described by others for larger sized microspheres [10, 22, 35]. Clearly, the simplicity of the modified solvent evaporation method, described in this study, allows for the efficient small scale production of appropriately sized PLGA or PLLA microspheres with high levels of PTX encapsulation. The in vitro release rate of PTX from either PLGA or PLLA microspheres was not determined in this study. Previous reports have demonstrated a slow PTX release from PLGA microspheres (less than 20% after 10 days) [12] and rapid release from low molecular wt PLLA microspheres (greater than 60% released after 10 days) [21].

The cyanogen bromide surface activation method for binding antibodies to polymer beads is a well established method in affinity chromatography protocols [13] and was used in this study to bind FITC-IgG antibodies in a concentration dependent manner (Figs. 3, 4). This method depends on the presence of hydroxyl groups on the surface of microspheres. PLGA and PLLA have only a small number of these surface groups. However, a multimolecular film of the emulsion stabilizer, PVA, remains incorporated in the surface of the microspheres even after washing and drying [18, 30]. For PVA, the hydrophobic backbone orients in the organic phase and the hydrophilic groups in the aqueous phase during the manufacturing process so the hydroxyl groups remain orientated to the outside of the microspheres after drying [18] and are available for antibody conjugation. These hydroxyl groups were sufficient to allow for excellent antibody conjugation. Initially, an orientation protein (Protein A) was bound to the microspheres before the IgG antibody was added (data not shown). Protein A allows for subsequent binding of the Fc region of IgG so that antibodies would be optimally orientated with the target binding moiety on the outer surface of the microsphere [13]. However, subsequent binding studies showed that this extra orientation step was unnecessary for effective microsphere binding to HUVEC cells so the use of Protein A was omitted.

For all cell culture and animal investigations PLLA microspheres were selected as the lead formulations over PLGA microspheres as PLLA degrades over just a few weeks and releases PTX more rapidly than PLGA. Therefore, we felt the drug release profiles might better suit the time frame of efficacy determinations (3 weeks). In this study, anti-VEGFR2 labeled, PTX-loaded microspheres were demonstrated to bind strongly to HUVEC cells in vitro (Fig. 5), confirming the rationale for the selection of the VEGFR2 receptor as the target binding site. The selection of the VEGFR2 antibody as the targeting moiety

was based on a growing body of literature that points to the importance of this receptor in angiogenic processes in prostate tumors. Indeed, following the identification of this receptor as the main angiogenic receptor for VEGF in endothelial capillary cells, a number of antiangiogenic agents that target this receptor or VEGF itself have been successfully developed [8, 11]. Whilst antiangiogenic agents may halt tumor growth, they are generally used in combination with chemotherapeutic agents that act in an additive or synergistic manner to kill tumor cells [8, 14, 19, 24]. These combination antiangiogenic and antitumor cell proliferation strategies have led to significant improvements in overall antitumor effects in comparison to the use of either agent alone. The use of PTX-loaded microspheres targeted to the vasculature of tumors described in this study may allow for the intratumoral controlled release of PTX which is both an antiangiogenic and antitumor agent.

The burst phase of drug release (large initial dose) for both types of microspheres (PLLA or PLGA) occurs during the first few days and does not depend on microsphere degradation but rather on drug diffusion from sources close to the surface of the microspheres. Since PTX inhibits tumor cell proliferation in the low nanomolar range (ng/ml) (16) and the 20 mg dose of PLLA microspheres release (at least) 10 μg PTX/ml/day (see Fig. 6) it seems likely that if even if only a small percentage of spheres bound to the tumor site then sufficient drug would be released daily to maintain a therapeutic dose. Degradation of these types of polyester polymers occurs at later time points so that most of the microspheres should remain bound to receptors before any detachment might occur. Therefore initial degradation processes do not automatically result in the drug depot breaking away from the antibody-receptor anchor.

Attempts were made to visualize Indocyanine Green labeled fluorescent microspheres in mice using a whole body animal imager. Although the microspheres could be clearly visualized following subcutaneous injection, systemically administered microspheres could not be located easily due to background autofluorescence encountered at high instrument sensitivity settings needed to detect microspheres in deeper tissues. When 20 mg of anti-VEGFR2 labeled microspheres were injected intravenously in mice bearing PC-3 tumors the fluorescent signal from the tumor area was weak. The strength of the fluorescent signal in the animal imaging system is dependent of the depth of the fluorescent source under the skin and the location of angiogenic zones in the tumors may vary depending on tumor morphology and depth under the skin making the animal imaging method subject to variation. Although some animals showed an apparent accumulation of anti-VEGFR2 labeled microspheres in the tumor sites the signal strength was not strong and the experiments were inconclusive. Further experiments are planned using radiological methods to measure microsphere accumulation in tumors.

When mice were injected with microspheres (20 mg intravenously), there was no evidence of poor tolerance or discomfort to the animals. In a pilot study, comparing unconjugated with anti-VEGFR2 conjugated, PTX-loaded microspheres there was clear evidence of the inhibition of tumor growth in the antibody targeting microsphere groups (Fig. 7). In this experiment the tumors were allowed to grow to larger sizes (more than 300 $mm^3$) than normally used in drug treatment studies [32]. The objective of this delayed treatment strategy was to allow for adequate angiogenesis and the expression of increased capillary cell binding sites for effective microsphere binding. Within the following weeks, some tumors grew rapidly and the animal studies experiment was terminated after 3 weeks according to the UBC Animal Care Committee ethical guidelines for animal tumor models. However, the inhibition observed in this model was statistically significant for a number of weeks and strongly suggests that the formulation represents a viable anticancer strategy for the treatment of prostate tumors. Future efficacy studies will include numerous controls such as non-microsphere-encapsulated PTX (free drug), unconjugated anti-VEGFR2 antibody (free antibody), these two together and lastly, microspheres with anti-VEGFR2 conjugated antibodies but no encapsulated PTX. This last control is quite important because we cannot rule out the possibility that the inhibition of tumor growth observed in this study did not arise, in part at least, from blockade of the VEGF receptors and inhibition of angiogenesis without any involvement of PTX. Future studies will also investigate earlier treatment schedules so that the tumors are smaller than those used in this study.

**Acknowledgments** The authors would like to thank the Canadian Prostate Cancer Research Initiative Ideas Grant program for providing funding for this project. The authors would also like to acknowledge Virginia Yago in Dr Gleave's group for performing the animal experiments.

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