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New platform for controlled and sustained delivery of the EGF receptor tyrosine kinase inhibitor AG1478 using poly(lactic-co-glycolic acid) microspheres

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Abstract

Inhibition of the epidermal growth factor receptor (EGFR) has been shown to reduce tumor growth and metastases and promote axon regeneration in the central nervous system. Current strategies for inhibiting EGFR include the administration of reversible or irreversible small-molecule tyrosine kinase inhibitors (TKIs). However, to be effective *in vivo* constant and sustained delivery is required. This study explored the feasibility of encapsulating the tyrphostin 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) in poly(lactic-co-glycolic acid) (PLGA) microspheres to achieve sustained delivery of the TKI. We characterized microspheres prepared using three different emulsion methods: solid-in-oil-in-water, oil-in-water, and oil-in-water with co-solvent. Addition of a co-solvent increased the loading and release of AG1478, and significantly ($P < 0.001$) decreased the size of the microspheres which facilitates administration of the spheres. On average, sustained delivery of AG1478 from microspheres was achieved for six months. However, the addition of a co-solvent prolonged release for over nine months (266 days). In addition, AG1478 retained its bioactivity upon delivery, and inhibited EGFR in both immortalized rat fibroblasts and in EGFR-amplified human carcinoma cells. These results demonstrate that AG1478 can be encapsulated in PLGA and retain bioactivity; thereby providing a new platform for controlled administration of EGFR TKIs.

Keywords

L epidermal growth factor receptor; tyrosine kinase inhibitors; AG1478; microspheres; PLGA

1. INTRODUCTION

The epidermal growth factor receptor (EGFR), also known as human EGF receptor 1 (HER1) or ErbB1, is one of four proteins in a family of transmembrane receptors with tyrosine kinase activity. Activation of EGFR with one of its seven ligands, such as epidermal growth factor (EGF) or transforming growth factor- α (TGF- α), leads to

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downstream regulation of cell proliferation, migration, apoptosis, and differentiation [1–3]. Overexpression of EGFR has been indicated in numerous pathologies, most notably in cancer [4–6], but also in nervous system diseases and injury [1, 7, 8]. Of these two pathologies, the role of EGFR in cancer has been extensively studied, and therapeutic strategies that target EGFR have been developed for anticancer treatments. Disrupting activation of EGFR through binding of TKIs has been shown to prevent cancer cell proliferation, invasion, metastasis, and neovascularization [6, 9].

Currently there are two small-molecule EGFR tyrosine kinase inhibitors (TKIs), which compete with ATP for binding to the intracellular kinase domain of the EGFR, that are FDA-approved for treatment of solid tumors, erlotinib (OSI774 or Tarceva®, OSI Pharmaceuticals) and gefitinib (ZD1839 or Iressa®, Astra Zeneca) [9]. While these drugs have proven successful as part of an anticancer therapeutic regimen, they come with significant side effects to the patient. Seventy percent of treated patients experience rashes in the form of acneiform eruption [10], and 54% of patients experience diarrhea [11].

While much focus has been placed on the role of EGFR in cancer, there is mounting evidence that EGFR also plays a significant role in modulating the post-injury environment in the central nervous system (CNS). Activation and upregulation of EGFR occurs in astrocytes after CNS injury and in neurodegenerative diseases, causing astrocytes to adopt a reactive phenotype [12] leading to formation of a glial scar [13], which prevents axon regeneration [14, 15]. EGFR affects not only morphological changes in astrocytes, but also causes astrocytes to increase production of axonal-growth inhibitory chondroitin sulfate proteoglycans [16, 17]. Strategies for inhibiting EGFR activity may have therapeutic benefit for treating nervous system injuries, such as those to the spinal cord [18] and optic nerve [12, 16, 19], by modifying the glial scar and promoting axon regeneration [12, 16, 20].

One of the particularly potent inhibitors for targeting EGFR is the hydrophobic EGFR TKI 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478). Administration of AG1478 leads to significant nerve regeneration [16] and increased neuronal survival [12]. However, the means of administration for AG1478 in these studies were inefficient with a large propensity for side effects, highly invasive, and costly. Previous work with AG1478 in preclinical trials emphasizes the need to maintain sustained levels of the drug to effectively inhibit EGFR [21]. However, due to its short circulation time, 30–48 min [21], administration requires a constant i.v. infusion to maintain therapeutic levels in the plasma. A similar challenge for treating solid tumors with TKIs. Thus, a system that delivers the inhibitor in a sustained, controlled, minimally invasive, and local manner is needed. In addition, this form of delivery may also provide a means to mitigate toxic side effects.

Biodegradable microspheres and nanospheres are excellent candidates for controlled release drug delivery systems *** I think you might want to be a bit more specific since you're going to a microsphere journal—they are excellent candidates for this drug because they permit similar, injectable administration and sustained delivery of a range of compounds. In particular, devices composed of poly(lactic-co-glycolic acid) (PLGA) have proven to be exceptional carriers for large and small molecule pharmaceuticals [22]. PLGA and its homopolymers, poly(glycolic acid) (PGA) and poly(lactic acid) (PLA), are biocompatible

and have been used in FDA-approved devices for 30 years [23–25]. Thus, use of these materials would facilitate clinical translation. For these reasons, we examined the feasibility of encapsulating the quinazoline AG1478 in PLGA microspheres. Quinazolines are a family of structurally similar tyrophostins that have shown the most promise in clinical trials as anticancer agents [9, 26, 27]. AG1478 was chosen due to its hydrophobicity, specificity for EGFR, potency *in vitro* ($IC_{50} = 3$ nM) [28], and its successful application in cancer [21, 29, 30] and CNS injury models [12, 16, 18].

Here we present the sustained delivery of AG1478 from PLGA microspheres. The release kinetics of the microspheres were tailored by varying fabrication parameters, which included the emulsion type and the incorporation of a co-solvent during fabrication. Encapsulation of AG1478 did not alter its bioactivity or its effectiveness when tested in FR3T3 fibroblasts and human A431 carcinoma cells. Development of these devices and establishing their effectiveness creates a new platform for delivery of AG1478 and other EGFR TKIs.

2. MATERIALS AND METHODS

2.1. Materials

Poly(lactic-co-glycolic acid) (PLGA) Resomer 503H (50:50 lactic to glycolic acid ratio and i.v. 0.32–0.44 dL g⁻¹) was obtained from Boehringer Ingelheim (Ingelheim, Germany). H signifies PLGA terminated with a carboxylic acid group. Poly(vinyl alcohol) (PVA) (MW ~25 kDa) was obtained from Poly Sciences (Warrington, PA). AG1478 (MW = 315.8 g mol⁻¹) was purchased from EMD Biosciences (San Diego, CA). Dichloromethane (DCM, A.C.S. grade), trifluoroethanol (TFE, A.C.S. grade), and dimethylsulfoxide (DMSO, A.C.S. grade) were purchased from Sigma-Aldrich (St. Louis, MO). Complete EDTA-free Proteases Inhibitor Cocktail was purchased from Roche (Mannheim, Germany). Sodium orthovanadate (Na₃VO₄) was purchased from Sigma-Aldrich (St. Louis, MO). Alkaline phosphatase (AP) conjugated secondary antibodies and the 1-Step NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) reagent were purchased from Pierce Biotechnology (Rockford, IL). Mouse, cell-culture grade EGF was purchased from BD (Franklin Lakes, NJ). All other cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

2.2. Microsphere Preparation

Microspheres were prepared using a single emulsion solvent evaporation technique [31]. For the solid-in-oil-in-water (s/o/w) single emulsion, 500 µL of DMSO was added to 5 mg of AG1478. This suspension was added to 200 mg of PLGA 503H in 2 mL of DCM while vortexing. The PLGA-AG1478 suspension was then added dropwise to 4 mL of 5% (w/v) PVA while vortexing. This emulsion was added dropwise to 100 mL 5% PVA and stirred for 3 h. The microspheres were centrifuged, washed three times with deionized water, flash frozen, lyophilized, and stored at -20°C.

We also prepared microspheres using an oil-in-water (o/w) single emulsion. 500 µL of DMSO was added to 5 mg of AG1478 and then heated to 37°C in a water bath to dissolve the inhibitor. This solution was added to 200 mg PLGA 503H in 2 mL of DCM while vortexing. The PLGA-AG1478 solution was added dropwise to 4 mL of 5% PVA while

vortexing. This emulsion was added dropwise to 100 mL of 5% PVA and stirred for 3 h. The microspheres were collected and stored as described above.

In addition to the previous fabrication methods, we prepared microspheres using the o/w single emulsion technique with the addition of a co-solvent at a specific ratio. 500 μL of DMSO was added to 5 mg of AG1478 and then heated to 37°C in a water bath to dissolve the inhibitor. This solution was added to 200 mg PLGA 503H in a 2.5 mL mixture of DCM solvent and TFE co-solvent (1:5 DCM:TFE, v/v). The resulting PLGA-AG1478 solution was added dropwise to 4 mL 5% PVA while vortexing. This emulsion was added dropwise to 100 mL 5% PVA and stirred for 3 h. The microspheres were collected and stored as described above.

Blank microspheres were made under identical conditions for each preparation, except no AG1478 was added.

2.3. Calibration of UV Absorbance for Quantification of AG1478 Loading and Release

Plotting UV absorbance versus AG1478 concentration produced a calibration curve for quantification of AG1478. For release time points, a linear fit was produced from 0.39–100 μM (~ 0.12 – $31.6 \mu\text{g mL}^{-1}$) AG1478 in PBS ($y = 0.01x - 0.0085$, $r^2 = 0.9996$). For loading, a linear fit was produced from 0.19–99.0 μM (~ 0.06 – $31.3 \mu\text{g mL}^{-1}$) AG1478 in DMSO ($y = 53.774x + 0.011$, $r^2 = 0.9993$). Using these curves we determined loading and release characteristics for the microspheres.

2.4. AG1478 Microsphere Loading

To determine loading of AG1478 microspheres, 5 mg of AG1478 microspheres or blank microspheres were placed in 1.5 mL tubes. One milliliter of DMSO was added to the tubes and vortexed to completely dissolve the microspheres [32]. The concentration of AG1478 in the microspheres was determined using a SpectraMax M5 ultraviolet (UV) spectrophotometer (Molecular Devices, Sunnyvale, CA) at 330 nm [33]. Encapsulation efficiency was determined by dividing the actual amount of drug loaded into the microspheres by the amount of drug added to the emulsion preparation (5 mg).

2.5. AG1478 Release from Microspheres

In 1.5 mL tubes, 10 mg of AG1478 microspheres or blank microspheres were reconstituted in 1 mL of phosphate buffered saline (PBS, pH 7.4). Samples were prepared in triplicate. Mixtures were then incubated at 37°C on a rotating shaker (Barnstead/Thermolyne; Dubuque, Iowa). At specific time points—1, 5, and 8h and 1, 3, 5, and 7 days and once every 7 days afterward until no AG1478 could be measured—the mixture was centrifuged and the supernatant collected. One milliliter of PBS was added to the tubes to replace the supernatant, and the mixture was then vortexed to resuspend the microspheres. The tubes were returned to the shaker until the next time point. Collected supernatants were stored at -20°C until they could be analyzed using UV spectroscopy at 330 nm.

2.6. Microsphere Size and Morphology Characterization

The volume-weighted mean diameter of the microspheres from each batch was measured using a Beckman Coulter Multisizer 3 (Fullerton, CA) with a 50 μm aperture and a sample size of at least 5,000 microspheres. Morphology was examined with scanning electron microscopy (SEM). Samples were mounted and sputter coated with gold for 30 s at 40 mA. Micrographs were taken on a Philips XL-30 environmental SEM operating at 10 kV.

2.7. Determination of Bioactivity of AG1478 Released from Microspheres

To determine bioactivity of AG1478 we tested supernatants from release time points on Fisher rat 3T3 fibroblasts (FR3T3) [34] and human A431 epithelial carcinoma cells (American Type Culture Collection, ATCC, Manassas, VA). FR3T3 and A431 cells were maintained in high glucose DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (penicillin-streptomycin-amphotericin B), at 37°C, 5% CO₂.

Before bioactivity was assessed, we determined the responsiveness of the two cell lines to AG1478. Cells were grown to 90% confluency and serum-starved overnight in Opti-MEM. AG1478 was added to cells at concentrations ranging 0–40 μM (~ 0 –12.6 $\mu\text{g mL}^{-1}$) for 30 min [21] at 37°C, 5% CO₂. After 30 min, EGF (100 ng mL⁻¹) was added to the cells for 2 min. Cells were then collected and lysed in buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 1X Complete EDTA-free Protease Inhibitor, 1 mM Na₃VO₄). Protein concentration from cell lysates was determined using the Bio-RAD Protein Assay reagent and concentrated to at least 1–3 mg mL⁻¹ total protein. Ten or twenty micrograms of protein in sample buffer (150 mM Tris-HCl, 6% SDS, 30% glycerol, 25% 2-mercaptoethanol, 0.0001% bromophenol blue) were added to each lane on reducing 6% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose. The blots were then blocked with 5% nonfat milk in Tris Buffered Saline and Tween and incubated with rabbit polyclonal antibody against EGFR (Cell Signaling Technology, Danvers, MA, working dilution 1:500, IgG) or mouse monoclonal antibody against phosphorylated-EGFR (Cell Signaling Technology, Danvers, MA, Tyr 1068, working dilution 1:500, IgG). Blots were also incubated with mouse monoclonal antibody against alpha-tubulin (Invitrogen, Carlsbad, CA, working dilution 1:2000, IgG) as a loading control. Blots were then incubated with the appropriate AP conjugated secondary antibodies (working dilutions 1:2000) and developed using the 1-Step NBT/BCIP reagent in AP buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl₂•6H₂O). From these blots, IC₅₀ values of AG1478 were determined for each cell line.

Bioactivity was assessed by adding sterile supernatants from day 35 of release from one batch of AG1478 microspheres at concentrations equal to the IC₅₀ value to serum-starved cells for 30 min. After 30 min, EGF (100 ng mL⁻¹) was added to the cells for 2 min. Cells were collected and protein was concentrated as described above. Protein from cell lysates were electrophoretically separated on reducing 6% SDS-polyacrylamide gels and blotted as described above.

2.8. Data Analysis and Statistics

The dose response curve was produced by creating a digital back and white image of the blot, inverting the image, and measuring the gray mean value for each band using Adobe

Photoshop CS3 Extended (San Jose, CA). The mean values for each band were normalized to the positive control band. To determine the IC₅₀ data were fit to a sigmoidal dose-response (variable slope) function using Prism 4 (Graph Pad Software, La Jolla, CA). Relative bioactivity was quantified by creating a digital image of the blot and measuring mean gray values as described above. A one-way ANOVA was used to determine differences among groups, with a Tukey-Kramer post test to evaluate significance as appropriate. A P-value of $P < 0.05$ was considered statistically significant. Percent inhibition is expressed as mean $\pm$ standard error mean (SEM).

3. RESULTS

3.1. Loading of AG1478 Microspheres

Microsphere loading was dependent upon the single emulsion technique utilized to prepare each batch. Microspheres prepared using the o/w techniques resulted in higher loading, when compared to the s/o/w technique (Figure 1). Furthermore, modifying the o/w emulsion with the addition of a co-solvent resulted in an ~38% increase in loading compared to o/w without a co-solvent (20.9 μg per mg and 15.1 μg per mg, respectively), and an ~125% increase in loading compared to s/o/w (9.30 μg mg⁻¹).

3.2. Release of AG1478

All formulations of microspheres sustained release for at least six months, with the o/w emulsion with co-solvent releasing for over nine months (266 days) (Figures 2A and 2B). As expected, when comparing emulsion techniques, the amount of released drug was directly related to the amount loaded. The release kinetics of microspheres from all three formulations followed a typical triphasic release for PLGA microspheres, with an initial burst, followed by a lag phase, and a secondary apparent-zero-order phase [35]. However, the o/w emulsion with co-solvent resulted in ~1.7 times more AG1478 released after the first day when compared to the s/o/w and o/w emulsions. Comparing the percent of total AG1478 released after one day (Figure 2B) revealed that the increase in the initial burst of AG1478 from microspheres prepared using the o/w emulsion with co-solvent ($11.1 \pm 0.173\%$) was indeed significantly different to the amount of AG1478 released from microspheres prepared using the s/o/w emulsion ($P < 0.001$) and the o/w emulsion ($P < 0.01$) techniques (s/o/w = $15.3 \pm 0.936\%$ and o/w = $8.05 \pm 0.248\%$, mean $\pm$ SD).

3.3. Microsphere Sizing and SEM

The volume-weighted mean diameters of microspheres fabricated using the s/o/w and o/w emulsion were almost identical, $19.3 \pm 8.18 \mu\text{m}$ and $20.7 \pm 7.93 \mu\text{m}$, respectively (mean $\pm$ SD). However, the mean diameter of microspheres fabricated using the o/w emulsion with co-solvent was notably smaller ($2.56 \pm 1.90 \mu\text{m}$). Table 1 summarizes the mean diameters for all formulations.

SEM images confirmed the results obtained from the Coulter Multisizer and revealed microspheres that were heterogeneous in size with minimal aggregation and smooth surfaces. Figures 3A and 3B show AG1478 microspheres from the o/w emulsion and o/w emulsion with co-solvent formulations.

3.4. Determination of IC₅₀ in FR3T3 and A431 Cells

We determined the potency of AG1478 in two cell types, FR3T3 cells which express normal/low EGFR levels (~10,000 receptors per cell) [36], and A431 cells, which express EGFR at very high levels ($2-3 \times 10^6$ receptors per cell) [37, 38]. Cells were incubated with increasing concentrations of AG1478 (0–40 μ M, ~0–12.6 μ g mL⁻¹) following challenge with a dose of EGF that provided maximal stimulation. Figures 4A and 4B show the dose response curves for both cell types.

From these curves, the IC₅₀ values were interpolated; values for both cell types were similar, with only a slight difference in potency of AG1478 observed for a wide range of EGFR expression levels present in A431 cells versus FR3T3 cells (10.5 nM and 6 nM, respectively).

3.5. Bioactivity of Encapsulated AG1478

Western blot analysis was used to determine the bioactivity of AG1478 released from encapsulated versus non-encapsulated AG1478 on inhibition of EGF-induced EGFR tyrosine phosphorylation in FR3T3 and A431 cells. ***since reviewers struggled, you might include a note here noting the mechanism and how phosphorylation plays into it*** FR3T3 cells were treated with non-encapsulated AG1478 or supernatants from encapsulated AG1478 at the IC₅₀ value (6.0 nM, 1.9 ng mL⁻¹) for 30 min and then challenged with EGF. Western blot analysis (Figure 5) demonstrated that the encapsulated inhibitor retained bioactivity and that it was as effective as non-encapsulated inhibitor in cells expressing EGFR at normal levels ($89 \pm 4.5\%$ and $89 \pm 2.7\%$, respectively).

To determine whether the encapsulated AG1478 would also be effective against cells with EGFR overexpression, A431 cells were tested as above with encapsulated and non-encapsulated AG1478 at the appropriate IC₅₀ value (10.5 nM, 3.3 ng mL⁻¹). Our western blot results showed that the released encapsulated inhibitor was less effective compared to non-encapsulated inhibitor ($40 \pm 8.3\%$ and $55 \pm 3.5\%$, respectively); however, a significant reduction in the level of EGFR tyrosine phosphorylation was still observed compared to untreated cells. In addition, we have also tested encapsulated AG1478 from later release time points and we have confirmed its bioactivity in FR3T3 cells, demonstrating that this encapsulation system remains effective until the polymer has completely degraded and all drug has been released.

4. DISCUSSION

In this study, we demonstrate for the first time, the successful encapsulation and long-term release of a bioactive EGFR TKI from PLGA microspheres ***has it been delivered in a sustained manner from other systems?***. We further show that a modification of our initial emulsion method extended this delivery for over six months. The ability to encapsulate small molecule inhibitors, such as this hydrophobic EGFR TKI, may provide a new avenue for the sustained delivery of therapeutic agents which have relatively short half-lives in patients, and may also minimize toxic side effects that are associated with high doses.

4.1. Loading, Sizing, and Release of AG1478 Microspheres

We observed an average AG1478 encapsulation efficiency of ~49% using the three emulsion methods, with o/w co-solvent having the greatest (66%) and s/o/w the least (29%). Changing the emulsion technique from s/o/w to o/w emulsion allowed the drug to fully dissolve in the solvent versus being in suspension, thereby facilitating an electrostatic interaction between the amine groups on AG1478 bonding with the carboxylic acid end groups in the PLGA. As expected, employing a co-solvent to the o/w emulsion also resulted in an increase in encapsulation (~125% and ~38% for s/o/w and o/w emulsions, respectively). Addition of TFE or other more hydrophilic solvents as a co-solvent in the emulsion process has been shown to increase the stability of the encapsulated drug in the oil phase and thus decrease the amount of drug lost in the aqueous phase [22]. While we did not vary the amount of AG1478 added during our fabrication, our results suggest that it may be possible to titrate the loading of AG1478 in PLGA to achieve appropriate therapeutic doses (e.g., 10 μM). One means to increase the loading is to use a water-miscible co-solvent, such as methanol or acetone [22].

In addition to affecting the encapsulation efficiency, use of TFE as a co-solvent also led to a significant ($P < 0.001$) decrease in the size of the microspheres. Microspheres fabricated using the s/o/w or o/w emulsion generally produced spheres on the order of 20 μm , approximately eight times the size of microspheres produced using the co-solvent method. Again, this was to be expected given that the addition of solvent such as TFE adds more energy to the system, thus decreasing the size of the microspheres [39]. This effect is further emphasized by the fact that fully dissolving AG1478 in the solvent prior to emulsification (e.g., o/w emulsion) did not result in a decrease in microsphere size. Therefore, it was the addition of the co-solvent that led to this effect.

Comparison of the release profiles of the three emulsion methods revealed a direct relationship between the encapsulation method and the amount released. As the solubility of the drug increased, i.e., from the s/o/w emulsion to the o/w emulsion, the amount of drug encapsulated (from 10.8 $\mu\text{g mg}^{-1}$ to 13.5 $\mu\text{g mg}^{-1}$) and released (Figure 2A) increased. The other effect observed involved the significant increase in the initial burst after one day of release in the o/w co-solvent compared to the other emulsions. This increase in burst is a result of the significant reduction in the size of the microspheres. Smaller sphere size increases the number of spheres per unit volume and thus increases the surface area, leading to more drug released in a shorter period. While the o/w co-solvent exhibited an increased burst, this method sustained the longest release of AG1478—over nine months (266 days). Furthermore, the amount of drug released at later time points in all fabrication methods were on average greater than 3 μM (~9.5 ng per mg polymer), an amount more than sufficient to achieve greater than 95% inhibition in the FR3T3 and A431 cells, as well as other cancer cell types [21, 30].

4.2. Bioactivity of Encapsulated AG1478

As a means to gauge the effectiveness of our system we tested it *in vitro* in FR3T3 and A431 cell cultures. We chose these two cell types in particular due to their EGFR expression levels. Using cell types that have vastly different receptor numbers allowed us to determine

effectiveness not only for normal EGFR expression, but for aberrant overexpression as well, which is often present in cancer cells [40]. In both cell types, administration of ~10 μ M (~3.2 μ g per mL) AG1478 approached 100% inhibition. This concentration matches the minimum value (10 μ M) required to completely inhibit EGFR in mouse skin and brain samples [21]. Ten micromolar is also the concentration that leads to robust optic nerve regeneration in a crush injury model [16].

Our system proved as effective as the non-encapsulated drug in FR3T3 cells, producing identical percent inhibition values. This finding demonstrates not only the efficacy of our system, but it also shows that encapsulating AG1478 did not significantly affect its bioactivity. Application of our system in A431 carcinoma cells did reveal a difference in percent inhibition between encapsulated and non-encapsulated AG1478; however, this reduction in inhibition, was not significant.

One advantage of our system versus the current means of administration of EGFR TKIs is the ability to deliver these drugs directly to tumors or sites of nerve injury via a needle injection, thereby providing an alternative route of administration for EGFR inhibitors [41]. Because our system provides sustained release of the drug, administration would be needed only periodically. Another important advantage of our system is the ability to administer a lower effective dose of TKIs, and thus reduce the toxic side effects observed from high-dose oral administration. While our work focused only on encapsulating AG1478, it can be assumed that encapsulation of other hydrophobic quinazoline tyrophostins, such as PD168393, would be feasible and produce similar characteristics observed with AG1478: efficient encapsulation, sustained delivery for at least six months, and little loss in bioactivity.

5. CONCLUSION

This study demonstrates the feasibility of encapsulating an EGFR TKI in PLGA. By manipulating the fabrication parameters we were able to tailor the encapsulation and release of AG1478 and the size of the microspheres. Addition of a co-solvent resulted in an increase in encapsulation and a significant decrease in microsphere size. It was determined that AG1478 remained bioactive after encapsulation and subsequent release from microspheres. Supernatants from release time points effectively inhibited tyrosine phosphorylation of EGFR *in vitro*. Further studies are needed to determine the efficacy of this system *in vivo* in mouse tumor models and in rat models of CNS injury.

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Figure 1. AG1478 loading for PLGA microspheres. Changes in the emulsion method resulted in increased encapsulation of AG1478, with the o/w co-solvent method producing the highest loading at $20.9 \mu\text{g mg}^{-1}$. Percentages above each bar represent encapsulation efficiency.

Figure 2.

Cumulative released (a) and cumulative released (percent of loading) (b) of AG1478 from PLGA microspheres prepared using the s/o/w (x), o/w (□), and o/w co-solvent (●) emulsions. Drug release of at least 4 months was retained for all emulsion methods. Total drug release <100% was due to polymer loss during supernatant extraction during release study. Data expressed as mean $\pm$ SD (n=3).

Figure 3. SEM micrographs of fabricated AG1478 microspheres. Microspheres fabricated using the o/w emulsion (A); microspheres fabricated using the o/w co-solvent emulsion (B).

Figure 4.

AG1478 dose response curves for FR3T3 (A) and A431 (B) cells displaying similar IC_{50} values. Cells were exposed to AG1478 at increasing concentrations from 0–40 μM (~ 0 –12.6 $\mu\text{g mL}^{-1}$) for 30 min, then challenged with EGF (100 ng mL^{-1}) for 2 min. Cell lysates were collected and equal amounts of protein were blotted for EGFR and p-EGFR as described in the methods. IC_{50} values were calculated by fitting the data to a sigmoidal dose-response function (dashed line).

Figure 5.

Western blots for EGFR and p-EGFR of cell lysates from FR3T3 and A431 cells treated with supernatants from microspheres (Enc AG, encapsulated AG1478), non-encapsulated AG1478 (AG), or no AG1478 (No AG, control) confirming bioactivity of AG1478 after encapsulation. Encapsulated AG1478 was as effective as non-encapsulated AG1478 in FR3T3 cells and A431 cells. α -Tubulin is used as the internal loading control. Each experiment was performed in triplicate.

Table I

Volume-weighted diameters of microspheres.

	AG1478 (μm)	Blank (μm)
Emulsion Method		
s/o/w	19.3 ± 8.18	27.0 ± 15.8
o/w	20.7 ± 7.93	26.2 ± 11.8
o/w co-solvent	2.56 ± 1.90	2.58 ± 1.96

Values: mean $\pm$ SD