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ARTICLE

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The application of electrosprayed minocycline-loaded PLGA microparticles for the treatment of glioblastoma

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Abstract

The survival of patients with glioblastoma multiforme (GBM), the most common and invasive form of malignant brain tumors, remains poor despite advances in current treatment methods including surgery, radiotherapy, and chemotherapy. Minocycline is a semi-synthetic tetracycline derivative that has been widely used as an antibiotic and more recently, it has been utilized as an antiangiogenic factor to inhibit tumorigenesis. The objective of this study was to investigate the utilization of electrospraying process to fabricate minocycline-loaded poly(lactic-co-glycolic acid) (PLGA) microparticles with high drug loading and loading efficiency and to evaluate their ability to induce cell toxicity in human glioblastoma (i.e., U87-MG) cells. The results from this study demonstrated that solvent mixture of dicholoromethane (DCM) and methanol is the optimal solvent combination for minocycline and larger amount of methanol (i.e., 70:30) resulted in a higher drug loading. All three solvent ratios of DCM:methanol tested produced microparticles that were both spherical and smooth, all in the micron size range. The electrosprayed microparticles were able to elicit a cytotoxic response in U87-MG glioblastoma cells at a lower concentration of drug compared to the free drug. This work provides proof of concept to the hypothesis that electrosprayed minocycline-loaded PLGA microparticles can be a promising agent for the treatment of GBM and could have potential application for cancer therapies.

KEYWORDS
drug delivery, electrospraying, glioblastoma, minocycline, PLGA microparticles

1 | INTRODUCTION

Glioblastoma (glioblastoma multiforme [GBM]) is the most common malignant brain and other central nervous system (CNS) tumor (14.3% of all tumors and 49.1% of malignant tumors) and is one of the most aggressive and high-grade form of these type of malignancies (Ostrom et al., 2021). It is a World Health Organization grade IV brain tumor which is associated with low overall survival of patient (Baid et al., 2020). There were 61,699 of newly diagnosed GBM tumors reported from 2014 to 2018, which averaged to be around 12,340 each year and equated to 3.23 per 100,000 people of the US population (Ostrom et al., 2021). The cases were projected to reach 13,160 and 13,430 per year for 2021 and 2022, respectively (Ostrom et al., 2021).

Despite treatment with surgery, radiotherapy and chemotherapy, the median survival remains poor (15 months median survival time, 5-year survival of ~5% after initial diagnosis) (Chang et al., 2016). The current method of treatment of gliomas are resection surgery, and

external beam radiation and occasionally systemic chemotherapy or a combination of these methods (Westphal et al., 2003). Metastasis of the tumor to other sites outside of the CNS does not occur very frequently for brain cancer, and thus, local recurrence is usually the main cause of death for patients with glioma (Manome et al., 2006). Due to the infiltrative nature of these tumors, surgeons often time are unable to completely remove all the cancer cells without the risk of resulting in neurological deficits (Marko et al., 2014). Thus, an emphasis should be placed on finding advances in treating the residual disease to prevent local recurrence in brain cancer patients after tumor resection to increase the long term survival or possibly the complete cure of patients with glioma (Lorger, 2012).

Minocycline (7-dimethylamino-6-dimethyl-6-deoxytetracycline) is a semi-synthetic tetracycline (Yong et al., 2004) derivative that has been widely used as an antibiotic for acne vulgaris, perioral disease and cutaneous sarcoidosis (Garrido-Mesa et al., 2013). The antibiotic properties of tetracyclines have been known since the late 1940s, however, more recently, tetracyclines such as minocycline have been identified for their other benefits including their antiapoptotic and anti-inflammatory activity and immunomodulatory and neuroprotective properties and thus, are beneficial for many diseases including osteoporosis, autism, AIDS, allergic asthma, atherosclerosis, ischemia, spinal cord injury, neuropathic pain, amyotrophic lateral sclerosis, multiple sclerosis and neuro-degenerative diseases (Garrido-Mesa et al., 2013). Additionally, minocycline has been utilized as an antiangiogenic factor to inhibit tumorigenesis (Bow et al., 2014; Frazier et al., 2003; Weingart et al., 1995) by inhibiting matrix metalloproteinases (Frazier et al., 2003); a group of metal-dependent enzymes that break down extracellular matrix proteins. Jung et al. (2014) demonstrated that minocycline prevent angiogenesis by inhibiting hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor that activates the transcription of genes that are involved in angiogenesis in cancer. Minocycline has also been shown to have the ability to induce glioma cell death through apoptosis and autophagy by endoplasmic reticulum stress (Liu et al., 2011, 2013).

Many chemotherapy and other antineoplastic agents, which can inhibit or halt the progression of tumors, such as immunotherapy or antiangiogenic drugs, have been investigated for the treatment of cancer through systemic delivery of these drugs. However, this delivery method is often associated with many disadvantages including low local drug concentration at the targeted site and nontarget cell and organ toxicity. Due to the short half-life of drugs, systemic delivery usually results in low efficacy of the delivered drug. Thus, there is a need to find alternative approaches to replace this current method of delivering drugs to treat cancer. Furthermore, brain malignancies must face an added challenge. Drugs for the treatment of brain tumors also must face the obstacle of crossing the Blood Brain Barrier (BBB). To overcome these challenges, biomaterials can be utilized to deliver drugs and their properties can be tailored to result in optimal drug concentration, drug administration schedule and sequence of delivery and exposure time. Microparticles fabricated from biomaterials can be used to deliver drugs to discrete areas of the body by stereotaxis and thus, will not require open surgery for implantation such as with biomaterial scaffolds (Xie et al., 2006). Delivering drugs locally with biomaterials can help ensure the drug concentration at the tumor environment is maximized, nontarget systemic exposure and organ toxicity is minimized and avoid the need of finding a method to cross the BBB. Local delivery of drugs can bypass the harsh environment and longer journey systemic delivery must take to reach the targeted site of interest, therefor resulting in an increased efficacy of the drug at the tumor site.

Poly(lactic-co-glycolic acid) (PLGA) is a α-hydroxy acid-derived polyester copolymer that is widely used as a biomaterial for drug delivery due to its inert properties and great biocompatibility. PLGA was approved by the US Food and Drug Administration for the bioresorbable surgical sutures in the 1970s, in 1986 approved for Decapeptyl® SR, the first PLGA microparticle-based product, and since then, more than 15 such microparticle products have been approved for the use in the clinical setting (Blasi, 2019). PLGA is a versatile polymer as its degradation rate can be easily tailored from days to years by controlling the lactic to glycolic acid ratio, molecular weight, and the end cap groups of the polymer. Furthermore, the biocompatibility of PLGA in the brains of rodents and humans has been demonstrated in previous studies (Kou et al., 1997; Menei et al., 1993). PLGA microparticles can be fabricated using different methods including emulsion solvent extraction/evaporation (M. Shi et al., 2010), electrospraying (Arya et al., 2009) and microfabrication (Acharya et al., 2010). Many groups utilized the emulsion solvent extraction/evaporation method for the fabrication of drug loaded microparticles as it a fabrication method that has an easy and low cost setup (Freiberg & Zhu, 2004; Li et al., 2008; Saralidze et al., 2010). However, with this technique, it is difficult to obtain high drug loading and entrapment efficiency as it often results in a large loss of drug to the surrounding solutions during the fabrication process (Acharya et al., 2010). To overcome this challenge, a group saturated the aqueous emulsion phase with 0.5 wt/vol % of drug (i.e., 400 mg of drug per synthesis of a standard batch of particles) (Zhang et al., 2012). Although able to result in high drug loading and entrapment efficiency, this saturation method requires a lot of drug which can be very costly for the fabrication process.

Minocycline is a hydrophilic drug due to its amino groups and thus, presents a challenge for encapsulation in a hydrophobic polymer and solubility in hydrophobic solvents and results in fast release rates due to its ability to dissolve easily in water (Holmkvist et al., 2016). Compared to hydrophobic drugs, hydrophilic drugs tend to be harder to encapsulate as they readily enter the aqueous phase before the PLGA chains form into particles when the typical emulsion solvent evaporation method is employed (Ramazani et al., 2016). Minocycline is also associated with instability at low or high pH, heat and exposure to light as it undergoes epimerization and results in degradation, which adds to the difficulty in encapsulating this drug and maintaining its bioactivity (Chow et al., 2008; Zbinovsky & Chrekian, 1977). We have previously utilized electrospraying to fabricate temozolomide-loaded PLGA microparticles for the treatment of glioma (Rodriguez de Anda et al., 2019). Typically, an electrospraying setup consists of a pump, a syringe and needle with

high electric potential and a grounded electrode. A high voltage potential is applied on the needle to force the polymer solution out of the syringe. A jet is formed that breaks into monodisperse droplets, which are collected on a copper plate as the solvent evaporates as it travels to the plate. The electrospraying fabrication method is very advantages as it results in particles with narrow size distribution, requires a low investment experimental setup and is easily scalable (Nguyen et al., 2016; Sridhar & Ramakrishna, 2013). This technique can also result in high loading efficiency compared to the common emulsion solvent extraction/evaporation technique (Sridhar & Ramakrishna, 2013), as shown in our previous work with temozolomide (TMZ) (Rodriguez de Anda et al., 2019).

The objective of this study was to investigate the utilization of electrospraying to fabricate minocycline-loaded PLGA microparticles with high drug loading and loading efficiency and to evaluate their ability to induce cell toxicity in human glioblastoma (i.e., U87-MG) cells. To the best of our knowledge, this is the first manuscript that investigated the electrospraying method for the fabrication of minocycline-loaded PLGA microparticles and evaluated them on human glioblastoma cells. There is only one previous work on electrosprayed minocycline-loaded PLGA microparticles, however, the particles were evaluated for periodontitis treatment (Zhang et al., 2021). Other prior research on minocycline-loaded PLGA microparticles or nanoparticles were fabricated using the emulsion solvent evaporation or nanoprecipitation techniques.

2 | MATERIALS AND METHODS

2.1 | Materials

U-87 MG glioblastoma cells (ATCC® HTB-14™) were obtained from American Type Culture Collection. Dulbecco's modified Eagle media (DMEM) with high glucose, L-glutamine and sodium pyruvate was purchased from Caisson's Lab (Smithfield, UT). Fetal bovine serum (FBS) Premium Select was obtained from Atlanta Biologicals. Penicillin-streptomycin was obtained from GE Healthcare Life Sciences HyClone Laboratories (Logan, UT). Methyl tetrazolium (MTT) powder, dichloromethane (DCM), isopropanol, acetone (ACE), acetonitrile (MeCN), Methanol (MeOH) and ethanol (EtOH) were obtained from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific. PLGA 75:25 (LP-1012, Internal Viscosity = 0.39 dL/g, MW = 54 kDa) with an acid end group was obtained from Evonik Industries. Minocycline Hydrochloride was purchased from TCI America.

2.2 | Fabrication of electrosprayed PLGA microparticles

PLGA microparticles loaded with minocycline were fabricated by electrospraying. The electrospraying setup consists of a Legato 100 Single Syringe Pump (KD Scientific), ES30P-5W power supply (Gamma High Voltage Research), a petri dish (100 × 15 mm) containing 60 mL isopropanol for microparticle collection and a glass syringe with a 21-gauge blunt needle tip (Hamilton). An 18 cm needle-tip to petri dish distance was used. A pump speed of 2 mL/h was used with a voltage of 9 kV. Briefly, 50 mg PLGA (5.0 wt/vol %) and the amount of drug shown in Table 4 were dissolved in a total of 1 mL of solvent. The amount of drug used for fabricating the microparticles was based on the maximum amount of minocycline that would dissolve into the different solvent combinations (i.e., 4, 8, and 16 mg in 1 mL of solvent for 90:10, 80:20, and 70:30, respectively). The particles were fabricated with a temperature and humidity range of 22°C–24°C and 45%–50%, respectively. The electrosprayed microparticles were collected in a petri dish containing isopropanol. For the "decant" method of collection, the isopropanol was decanted immediately after fabrication and the remaining solvent was allowed to evaporate for 24 h. For the "evaporation" method of collection, the isopropanol was allowed to evaporate by itself for 24 h. The collected particles were frozen (−20°C) for 24 h and freeze-dried under vacuum for 24 h to ensure complete removal of the solvent.

2.3 | Drug loading and loading efficiency

The amount of drug loaded into the microparticles was determined using a previous protocol with some modifications (Zhang et al., 2012). A total of 2 mg of microparticles were completely dissolved in 1 mL of DMSO (100%). The amount of minocycline was determined by measuring the absorbance at 350 nm (Xing et al., 2012) with a microplate reader (BMG CLARIOSstar Plus). The drug loading and loading efficiency analyses were done in biological triplicates ($n = 3$) and technical triplicates ($n = 3$). The drug loading and loading efficiency were determined with the equations below.

$$\text{Drug loading (\%)} = \frac{\text{weight of drug in sample}}{\text{total weight of sample}} \times 100,$$

$$\text{Loading efficiency (\%)} = \frac{\text{actual amount of drug loaded}}{\text{theorectical amount of drug loaded}} \times 100.$$

2.4 | Drug release kinetics

A total of 3 mg of microparticles were placed in 1 mL of phosphate buffered saline (PBS) in a 1.5 mL microcentrifuge tube at 37°C and shaken at 70 rpm. At each time point (1 and 3 h, 1, 4, 7, 10, 14 days), the microparticles were centrifuged at 14,000 rpm for 10 min. and the amount of minocycline released into the PBS was obtained as described above. The particles were then replenished with 1 mL of fresh PBS. This study was done in biological triplicates ($n = 3$) and technical triplicates ($n = 3$).

2.5 | Imaging by optical microscopy and scanning electron microscopy (SEM)

Microparticles morphology and distribution were determined by optical microscopy using Zeiss Binocular Compound Microscope).

Microparticle surface morphology and distribution were also observed by SEM using a Zeiss Sigma VP Field Emission Scanning Microscope. SEM images were taken using an electron beam operating at up to 6 kV. The microparticles were sputter coated with a 5 nm gold layer using a smart coater (JEOL) to generate electrical conduction coating on the sample surfaces to increase their electrical conductivity. The PLGA microparticles were processed for the evaluation of morphology, size, and shape by analyzing images at different magnifications.

2.6 | Microparticle size determination

The diameter of the microparticles was determined from the SEM images using the NIH ImageJ Software. For each image, the diameter of particles where the whole particle could be clearly identified in the images (i.e., not covered by other particles) was measured.

2.6.1 | Fourier transform infrared spectroscopy (FTIR)

FTIR was performed using a Nicolet™ iS™ 5 FTIR Spectrometer to analyze the chemical composition of PLGA, minocycline, minocycline-loaded microparticles and the powder left after evaporating the decant solution. The spectrum was recorded at room temperature between 500 and 4000 cm^{-1} with a resolution of 1 cm^{-1}.

2.7 | Cell viability

An MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich) was used to analyze the cytotoxicity of minocycline in the human U-87 MG glioblastoma cell line as previously described for testing of microparticles (Zhang & Gao, 2007). The U-87 MG glioblastoma cells were cultured in DMEM media supplemented with 10% FBS, 100 U/mL penicillin and 100 µg streptomycin at 37°C, 5% CO_2 and 90% relative humidity. The cells were plated in a 96-well plate at a cell density of 1×10^4 cells/100 µL per well. After allowing the cells to attach overnight, the media in each well was replaced with 100 µL of media containing free minocycline, empty PLGA microparticles or minocycline-loaded PLGA microparticles. Wells containing only media were used as a control. After 72 h, an MTT assay was performed to determine the ability of the drugs to induce cytotoxicity in glioblastoma cells. Briefly, the media in each well was replaced with 100 µL of MTT solution at a final concentration of 0.5 mg/mL (Milipore Sigma, n.d.; Nga et al., 2020). After 3 h, the MTT solution was removed and 100 µL of DMSO was added to each well to dissolve the formazan salts that were formed by viable cells by metabolizing the MTT. The absorbance at 570 nm was determined with a microplate reader (BMG CLARIOSstar Plus). Four biological replicates ($n = 4$) were used for each condition and the following equation was used to determine cell viability (%).

$$\text{Cell viability (\%)} = \frac{\text{absorbance of test cells}}{\text{absorbance of control cells}} \times 100.$$

2.8 | Statistical analysis

Multiple-factor analysis of variance was conducted to identify if there were any significant differences among groups ($p < 0.05$) followed by the use of Tukey's Honestly Significantly Different test to identify the specific groups that differed statistically significantly.

3 | RESULTS

3.1 | Optimal solvents for electrospraying and collecting minocycline-loaded PLGA microparticles

To determine the optimal solvents to utilize for electrospraying and collection of the PLGA microparticles, a drug solubility study was performed with different solvents. As seen in Table 1, for minocycline, it was determined that methanol was able to dissolve the most drug (more than 44 mg in 1 mL of solvent) followed by DMSO (40 mg in 1 mL of solvent) and ethanol (30 mg in 1 mL of solvent). For PLGA, there were several solvents that were able to dissolve a high amount of the polymer at more than 50 mg in 1 mL of solvent: DCM, acetone, acetonitrile and DMSO. Alternatively, isopropanol was the solvent that was not ideal to dissolve both minocycline and PLGA (<1 mg in 1 mL of solvent). Looking at solvent combinations (Table 2), DCM:Methanol at a solvent ratio of 70:30 was able to dissolve more minocycline (30 mg in 1 mL of solvent) compared to other solvent combination tested (26 mg in 1 mL of solvent for DCM:DMSO, 1 mg in 1 mL of solvent for DCM:Ethanol and <1 mg in 1 mL of solvent for DCM:Acetone and DCM:Acetonitrile). For solvent ratio of 80:20 and 90:10, we investigated the solubility for the two highest combination solvents for 70:30 which were DCM:Methanol and DCM:DMSO. It was found that DCM:Methanol was able to dissolve more drug (8 and 4 mg in 1 mL of solvent for 80:20 and 90:10, respectively) compared to DCM:DMSO (2 and 1 mg in 1 mL of solvent for 80:20 and 90:10, respectively).

TABLE 1 Solubility of minocycline and PLGA in 1 mL of solvent.

Solvents	Amount of minocycline (mg)	Amount of PLGA (mg)	Vapor pressure (hPa)
DCM	<1	>50	475
Methanol	>44	<1	128
Acetone	<1	>50	240
Acetonitrile	1	>50	97
DMSO	40	>50	0.61
Ethanol	30	<1	59
Isopropanol	<1	<1	400

Abbreviations: DCM, dicholoromethane; DMSO, dimethyl sulfoxide; PLGA, poly(lactic-co-glycolic acid).

3.2 | Drug loading and loading efficiency

Table 3 summarizes the drug loading and loading efficiency of the four different types of microparticles evaluated during the optimization process. The drug loading for the evaporation collection method was significantly higher than the decant collection method for both the 2.5 (2.1 ± 0.0% vs. 0.9 ± 0.2%) and 4.0 mg (2.9 ± 0.3% vs. 1.6 ± 0.1%) drug amounts use in 1 mL of fabrication solution. When using a higher amount of drug for fabrication (i.e., 4.0 mg vs. 2.5 mg) a higher drug loading was achieved for both the decant (1.6 ± 0.1% vs. 0.9 ± 0.2%) and evaporation (2.9 ± 0.3% vs. 2.1 ± 0.0%). A higher loading efficiency was achieved when the evaporation method was used compared to the decant method when 2.5 mg (43.1 ± 0.6% vs. 17.9 ± 3.1%) or 4.0 mg (39.3 ± 3.8 vs. 21.7 ± 1.4%) was used for fabrication. The loading efficiencies were not significantly different when different amounts of drug were used for fabrication and the same method of collection was used.

When the different solvent ratios were investigated (Table 4), the 70:30 group had significantly higher drug loading (12.7 ± 1.0%) compared to 80:20 (4.4 ± 0.2%) and 90:10 (2.9 ± 0.3%) groups. The 70:30 group also had significantly higher loading efficiency (i.e., 52.3 ± 4.0%) compared to 80:20 (31.5 ± 1.7%) and 90:10 (39.3 ± 3.8%) groups.

TABLE 2 Solubility minocycline in 1 mL of a combination of two solvents.

Solvent ratio	Solvents	Amount of minocycline (mg)
70:30	DCM:Methanol	30
	DCM:Acetone	<1
	DCM:Acetonitrile	<1
	DCM:DMSO	26
	DCM:Ethanol	1
	DCM:Isopropanol	<1
80:20	DCM:Methanol	8
	DCM:DMSO	2
90:10	DCM:Methanol	4
	DCM:DMSO	1

Abbreviations: DCM, dicholoromethane; DMSO, dimethyl sulfoxide.

3.3 | Microparticle size and surface morphology

Overall, the mean diameter for the microparticles fabricated ranged from 62 to 88 µm. For both types of particles made with 2.5 or 4.0 mg of drug (Table 3), the sizes of the particles were significantly larger for the evaporation collection method (87.8 ± 26.5 and 75.5 ± 15.7 µm, respectively) compared to the decant collection method (72.6 ± 15.1 and 62.7 ± 16.0 µm, respectively). The amount of drug used for fabrication (Table 3) did not affect the size of the microparticles. For the different solvent ratios (Table 4), the sizes of all particles were not significantly different from each other and ranged from 76 to 80 µm.

Light microscopy (Figures 1 and 2, a1–a4) and SEM images (Figures 1 and 2, b1–b4 and c1–c4) of the different microparticles fabricated with different drug amount and collection methods (i.e., 90:10 DCM to Methanol with different 2.5 or 4.0 mg of drug and decant vs. evaporation collection method) and different solvent ratios (i.e., 70:30, 80:20, and 90:10 DCM to methanol) showed spherical microparticles. Close up images of each microparticles reveal smaller spheres present on each microparticles (Figures 1 and 2, b1–b4). Minocycline drug powder appears to be elongated and irregular in shape (Figure 3a). Minocycline that was dissolved in isopropanol and the isopropanol was allowed to evaporate looked crystalline in structure (Figure 3b). The substance that was collected after evaporating the decant solution appears to be small spheres (Figure 3c).

3.4 | Microparticles and decant powder composition

The FTIR spectra of PLGA, minocycline, minocycline-loaded microparticles and the powder left after evaporating the decant solution

TABLE 3 Electrosprayed minocycline-loaded PLGA microparticles parameters and drug loading (%), loading efficiency (%) and mean diameter (µm) of microparticles fabricated with different minocycline amounts and collection methods, but same DCM:Methanol ratio (90:10).

Group	Drug amount (mg in 1 mL)	Collection method	Drug loading (%)	Loading efficiency (%)	Mean diameter (µm)
2.5 mg_Decant	2.5	Decant	0.9 ± 0.2^{a}	17.9 ± 3.1^{a}	$72.6 \pm 15.1^{a,b}$
2.5 mg_Evaporation	2.5	Evaporation	2.1 ± 0.0^{b}	43.1 ± 0.6^{b}	87.8 ± 26.5^{c}
4.0 mg_Decant	4	Decant	1.6 ± 0.1^{c}	21.7 ± 1.4^{a}	62.7 ± 16.1^{a}
4.0 mg_Evaporation	4	Evaporation	2.9 ± 0.3^{d}	39.3 ± 3.8^{b}	$75.5 \pm 15.7^{b,c}$

Note: The results are expressed as mean ± standard deviation for $n = 3$ biological replicates and $n = 3$ technical replicates. Groups with different lowercase letters (i.e., a vs. b vs. c) are significantly different ($p < 0.05$) from one another within a particular category.

Abbreviations: DCM, dicholoromethane; DMSO, dimethyl sulfoxide.

TABLE 4 Electrosprayed minocycline-loaded PLGA microparticles parameters and drug loading (%), loading efficiency (%) and mean diameter (μm) of microparticles fabricated with different solvent ratios, but same collection method (evaporation).

Group	Solvent ratio DCM:Methanol	Drug amount (mg in 1 mL)	Drug loading (%)	Loading efficiency (%)	Mean diameter (μm)
Mino_90	90:10	4	2.9 ± 0.3[a]	39.3 ± 3.8[a]	75.5 ± 15.7[a]
Mino_80	80:20	8	4.4 ± 0.2[a]	31.5 ± 1.7[a]	76.9 ± 14.0[a]
Mino_70	70:30	16	12.7 ± 1.0[b]	52.3 ± 4.0[b]	79.5 ± 13.6[a]

Note: The results are expressed as mean ± standard deviation for $n = 3$ biological replicates and $n = 3$ technical replicates. Groups with different lowercase letters (i.e., a vs. b vs. c) are significantly different ($p < 0.05$) from one another within a particular category.

Abbreviations: DCM, dicholoromethane; DMSO, dimethyl sulfoxide.

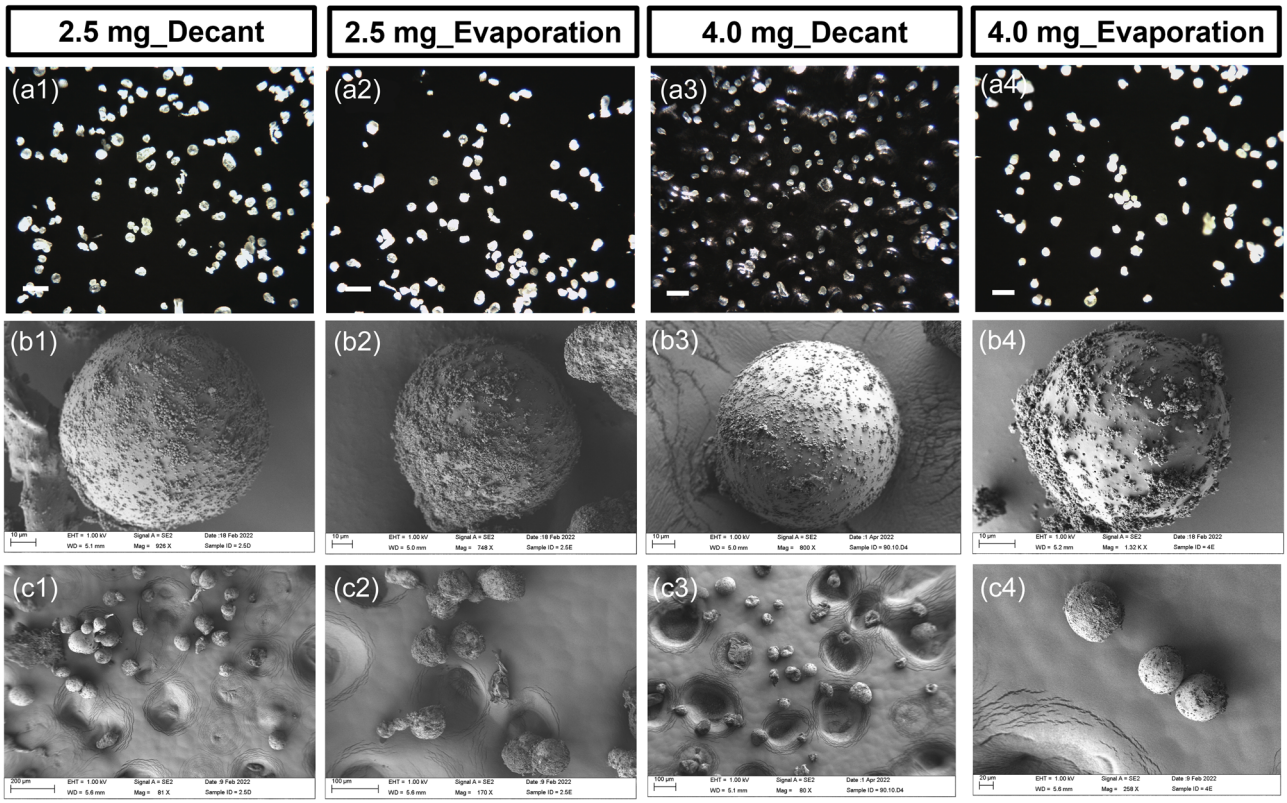

FIGURE 1 Light microscopy and SEM images of the electrosprayed minocycline-loaded PLGA microparticles fabricated with different drug amount and collection method: (a1–a4) Optical microscopy images (scale bar of these images represent 10 μm); (b1–b4) SEM images with high magnification: (b1) 2.5 mg_Decant, ×926, scale bar represents 10 μm, (b2) 2.5 mg_Evaporation, ×748, scale bar represents 10 μm, (b3) 4.0 mg_Decant, ×800, scale bar 10 μm, and (b4) 4.0 mg_Evaporation, ×1320, scale bar 10 μm; (c1–c4) SEM images with low magnification: (c1) 2.5 mg_Decant, ×81, scale bar represents 200 μm, (c2) 2.5 mg_Evaporation, ×170, scale bar represents 100 μm, (c3) 4.0 mg_Decant, ×80, scale bar 100 μm, and (c4) 4.0 mg_Evaporation, ×258, scale bar 20 μm. PLGA, poly(lactic-co-glycolic acid); SEM, scanning electron microscopy.

are shown in Figure 4. The PLGA sample had the characteristic $-CH_2$ and $-CH_3$ (2950 cm^{-1}), the –COOH (1747 cm^{-1}), and the C–O–C (1082 cm^{-1}) bands in PLGA as seen by others (Fu et al., 2017; Zhang et al., 2021). As seen by Zhang et al. (2021), the minocycline sample had the characteristic –COOH (1650 and 1600 cm^{-1}) and multiple complex peaks associated with the four benzene rings in minocycline (500–1400 cm^{-1}). The minocycline-loaded PLGA microparticles had both the PLGA and minocycline associated bands and peaks. The powder left after evaporating the decant solution also had bands and peaks that were associated with both the PLGA and minocycline.

3.5 | Drug release kinetics

The drug release kinetics of the microparticles fabricated with different DCM:Methanol ratios are shown in Figure 5. A burst released of the drug (i.e., 70%–87%) was observed for all the different groups at the first time point (i.e., 1 h) (Figure 5a). A significantly larger burst released was observed for 70:30 (87.20 ± 2.12%) compared to 80:20 (83.0 ± 0.9%) and 90:10 (80.1 ± 1.1%). The burst release for 80:20 (83.0 ± 0.9%) was also significantly larger than 90:10 (80.1 ± 1.1%). After 3 h, the % cumulative drug released for 70:30 was significantly higher (90.8 ± 1.8%) compared

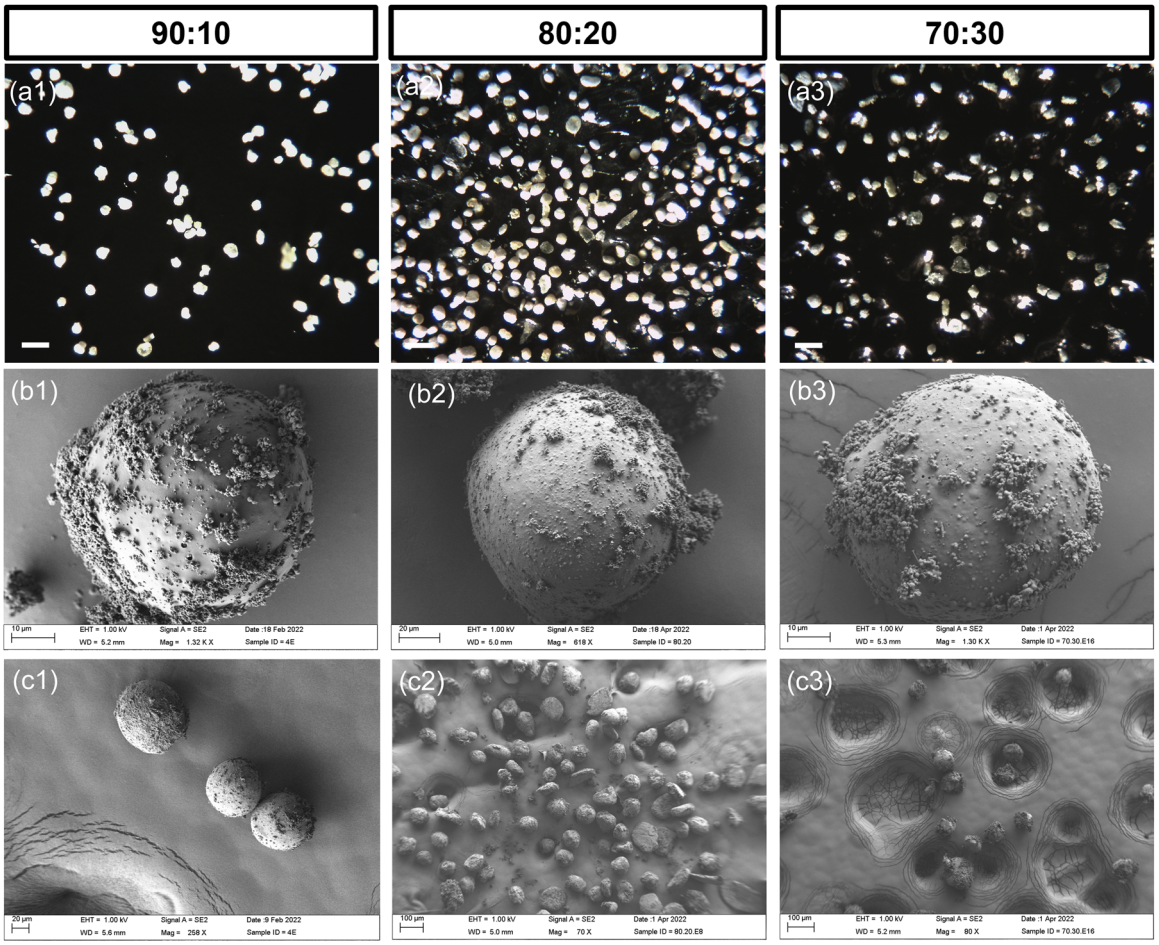

FIGURE 2 Optical microscopy and SEM images of the electrosprayed minocycline-loaded PLGA microparticles fabricated with different drug amount and collection method: (a1–a3) Optical microscopy images (scale bar of these images represents 10 μm); (b1–b3) SEM images with high magnification of: (b1) 90:10, ×618, scale bar represents 20 μm, (b2) 80:20, ×748, scale bar represents 10 μm and (b3) 70:30, ×1300, scale bar 10 μm; (c1–c3) SEM images with low magnification: (c1) 90:10, ×258, scale bar represents 20 μm, (c2) 80:20, ×70, scale bar represents 100 μm and (c3) 70:30, ×80, scale bar 100 μm. PLGA, poly(lactic-co-glycolic acid); SEM, scanning electron microscopy.

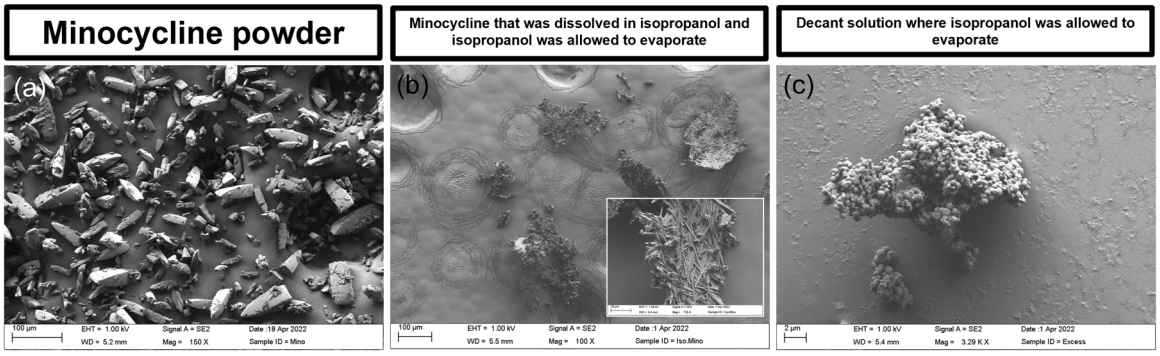

FIGURE 3 SEM images of (a) minocycline drug powder, ×150, scale bar represents 100 μm. (b) Minocycline that was dissolved in isopropanol and then the isopropanol was allowed to evaporate, ×1840, scale bar represents 3 μm (embedded picture: ×735, scale bar represents 20 μm) and (c) decant solution where the isopropanol was allowed to evaporate, ×3290, scale bar represents 2 μm. SEM, scanning electron microscopy.

to 80:20 (88.0 ± 1.0%) and 70:30 (85.5 ± 1.8%). The amount of drug released after the first time point (i.e., 1 h) for 70:30 was significantly higher (0.24 ± 0.04 mg) compared to 80:20 (0.12 ± 0.00 mg) and 90:10 (0.08 ± 0.00 mg) (Figure 5c).

When evaluating the drug release from 3 h onwards (i.e., omitting the burst release at 1 h due to the possibility of the release of the drug that is adsorbed onto the particles), the cumulative release at all timepoints for 90:10 were

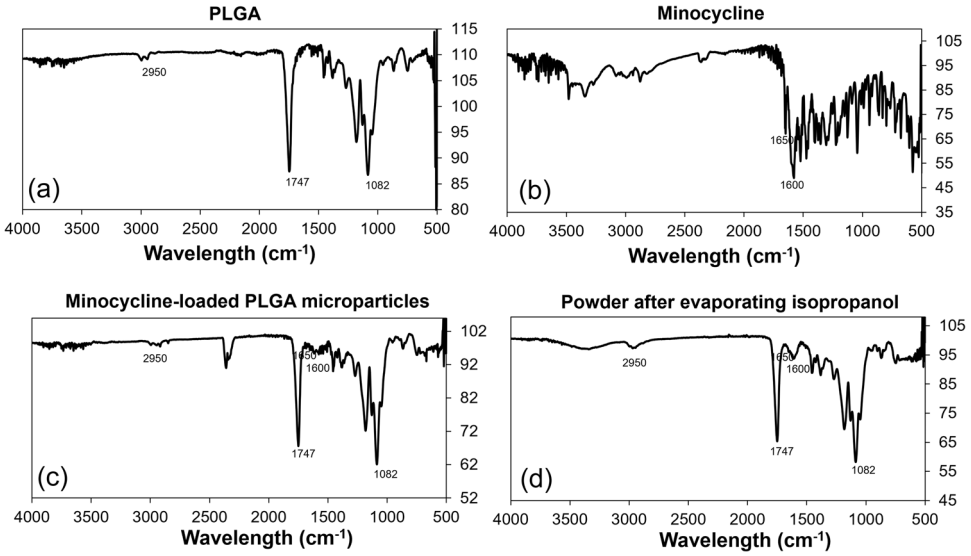

FIGURE 4 FTIR spectra of (a) PLGA, (b) Minocycline, (c) Minocycline-loaded PLGA microparticles and (d) Powder after evaporating isopropanol. FTIR, fourier transform infrared spectroscopy; PLGA, poly(lactic-co-glycolic acid).

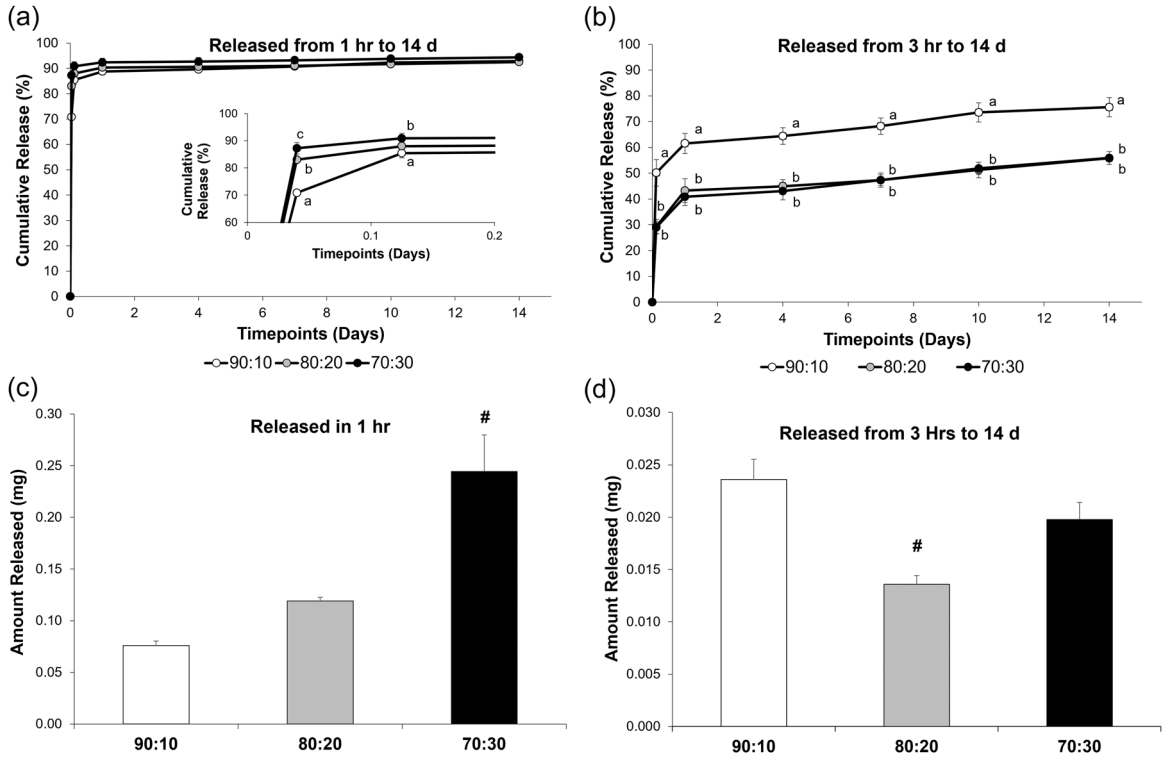

FIGURE 5 Cumulative release from (a) 1 h to 14 days and (b) 3 h to 14 days. Groups with different lowercase letters (i.e., a vs. b vs. c) are significantly different ($p < 0.05$) from one another within a particular category. Amount released (c) after 1 h and (d) from 3 h to 14 days. #Indicates groups that are statistically significantly different ($p < 0.05$) from all other groups. The results are expressed as mean ± standard deviation for $n = 3$ biological replicates and $n = 3$ technical replicates.

significantly higher (50%–76%) compared to 80:20 (29%-56%) and 70:30 (29%–56%) (Figure 5b). The amount of drug released from 3 h to 14 days was significantly lower for 80:20 (0.01 ± 0.00 mg) than 90:10 (0.02 ± 0.00 mg) and 70:30 (0.02 ± 0.00 mg) (Figure 5d).

3.6 | Cell viability

To evaluate the response of glioblastoma U-87 MG cells to minocycline alone, empty PLGA microparticles and minocycline-loaded PLGA microparticles, an MTT cell viability assay was

performed. The 70:30 microparticles were used as an initial proof-of-concept of the bioactivity of the drug loaded in the electrosprayed minocycline-loaded PLGA microparticles. The results demonstrated that empty PLGA microparticles did not elicit a negative response on cell viability of the U87-MG cells (Figure 6). However, U87-MG cells exposed to minocycline by itself at a concentration of 400 μM had a significantly lower % cell viability (i.e., 400 FD [FD = free drug], 78.3 ± 5.2%) compared to the control (100 ± 6.1%) and empty PLGA microparticles (i.e., 400B [B =blank], 92.1 ± 3.5%). Minocycline-loaded PLGA microparticles resulted in a significantly lower % cell viability at all the drug concentration tested (i.e., 200, 300, and 400 μM denoted as 200, 300, and 400 MP [MP = microparticles], 66.8 ± 3.9%, 48.7 ± 6.3%, and 44.9 ± 3.9%, respectively) compared to the control (100 ± 6.1%), empty PLGA microparticles (i.e., 200B, 300B, 400B), 97.0 ± 7.8%, 87.6 ± 6.7%, and 92.1 ± 3.5%, respectively) and minocycline by itself (i.e., 200, 300, and 400 FD), 101.1 ± 9.6%, 88.4 ± 11.9%, and 78.3 ± 5.2%, respectively). At a drug concentration of 300 and 400 μM, the minocycline-loaded PLGA microparticles resulted in a significantly lower % cell viability (300 and 400 MP), 48.7 ± 6.3% and 44.9 ± 3.9%, respectively) compared to minocycline-loaded PLGA microparticles at a drug concentration of 200 μM (i.e., 200 MP, 66.8 ± 3.9%).

4 | DISCUSSION

The emulsion solvent evaporation technique is a common fabricating method for PLGA microparticles as it is associated with low cost and ease in setup. However, one of the biggest challenges associated with this method is the high loss of drug to the external aqueous phase by diffusion, which results in low drug loading and loading efficiency of the final product. Thus, in this work, we investigated the application of the electrospraying technique to fabricate minocycline-loaded PLGA microparticles with higher drug loading and loading efficiency for the treatment of glioblastoma. The effects of altering the solvent used for the fabrication of the microparticles on drug loading, loading efficiency, and release kinetics, particle size and morphology, and the ability of the microparticles to elicit glioblastoma cell death were investigated.

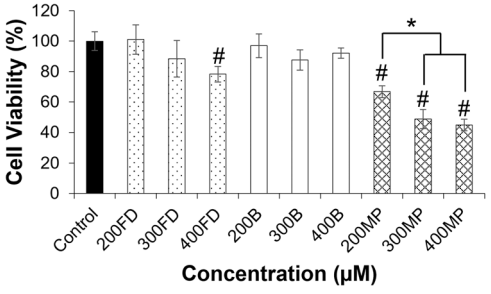

FIGURE 6 Cell viability of U-87 MG glioblastoma cells exposed to the minocycline only (denoted as FD), empty PLGA microparticles only (denoted as B) or minocycline-loaded PLGA microparticles (denoted as MP) for 72 h. The results are expressed as mean ± standard deviation for $n = 4$ biological replicates. #Indicates groups that are statistically significantly different ($p < 0.05$) from the control and empty PLGA microparticles within the same drug concentration. *Indicates statistically significantly different ($p < 0.05$) between groups indicated. FD, free drug; MP, microparticles; PLGA, poly(lactic-co-glycolic acid).

Finding the optimal solvent for electrospraying is key to successful fabrication of electrosprayed microparticles. Minocycline is a hydrophilic drug due to the amino groups in its structure and thus, is not very soluble in hydrophobic solvents (Holmkvist et al., 2016). Methanol was determined to be the most optimal solvent to dissolve minocycline and thus, was included as one of the solvents for fabrication to help increase the amount of drug that can be dissolved in the electrospraying polymer/drug solution and result in increased drug loading. The addition of methanol also resulted in an increase in loading efficiency (i.e., 70:30 had a significantly higher loading efficiency compared to 80:20 and 90:10). This could have resulted from the tendency of minocycline to be able to stay in solution when a higher amount of methanol is used, compared to when a lower amount of methanol is used which may results in precipitation of the drug in the syringe over time, resulting in less incorporation of the drug when the polymer/drug solution is electrosprayed. For PLGA, DCM, acetonitrile and DMSO were all able to dissolve a high amount of PLGA, however, DCM was chosen because of its higher vapor pressure (475 hPa) compared to the other solvents (97 and 0.61 hPa for acetonitrile and DMSO, respectively). A solvent with a higher vapor pressure exerts more pressure on the liquid phase resulting in higher volatility or ease of vaporization. Lower volatility of solvent can result in slower extraction of the solvent which can in turn lead to pores in the particles. As seen in our previous study (Rodriguez de Anda et al., 2019) where acetonitrile was used in combination with DCM for the fabrication of TMZ-loaded microparticles, pores were created as an artifact of the solvent evaporation process which can result in initial burst release of drugs due to pore diffusion (Mao et al., 2007; Yeo & Park, 2004). Rapid initial release of drug often occurs due to the hydration of the outer wall of the particles which leads to leaching of the drug (Freiberg & Zhu, 2004). Surface porosity of PLGA microparticles plays an essential role in determining the magnitude of this initial release due to pore diffusion (Mao et al., 2007). We demonstrated that larger pores due to higher amount of the less volatile solvent resulted in a high burst release compared to particles with small pores. Thus, to avoid burst release and to allow the particles to set as a sphere before hitting the collection plate or solution and avoid forming particles which are flat on one end, using a solvent with high volatility may be more ideal.

In our previous work, TMZ-loaded microparticles were successfully fabricated using acetonitrile and DCM by themselves or in combination by collecting the particles on a glass plate attached to a copper plate as the dried microparticles can be more easily removed from the glass. In this work, we were not able to fabricate the minocycline-loaded microparticles with spherical morphology and instead resulted in films or fused particles when the same collection

method was used. DCM is a volatile solvent, and thus can evaporate and result in the formation of discrete particles. However, it is not a good solvent for minocycline and thus, methanol had to be incorporated to result in higher incorporation of the drug in the polymer/drug solution. The incorporation of methanol, however, resulted in fused particles instead of discrete particles. The ideal volatility of the solvent is key in the shape and morphology of the collected particles. When the solvent is too volatile, the particles tend to be hollow and porous, and can lead to them collapsing when arriving at the plate (Zhang et al., 2019). When the solution volatility is low, the nice spherical shape may deform to semi-spherical particles (i.e., flat on one end) when they reach the collection plate. When there is residual solvent in the particles, aggregation and deformation of the particles or polymeric films can form (Zhang et al., 2019). Thus, a solvent with medium vapor pressure may be ideal. Besides vapor pressure, the solution viscosity (which can change with the type of drug and amount of drug dissolved in the solvent), surface tension and electrical conductivity also plays a role in the success of electrospraying particles with ideal morphology (Zhang et al., 2019) and, therefore, although the DCM by itself worked for TMZ in our previous work (Rodriguez de Anda et al., 2019), it may not work for minocycline. Thus, in this work, we investigated the utilization of a liquid collector (i.e., collection media) instead of a plate to collect the microparticles. By collecting the microparticles in isopropanol, discrete particles were obtained instead of film as when collected on a flat plate collector (Nguyen et al., 2016; Uhl et al., 2020). Furthermore, the utilization of a liquid collector where discrete particles can be collected is beneficial as it is associated with the relative ease of scale-up (Uhl et al., 2020).

For the collection of the microparticles in a liquid media, a solvent that is not a good solvent (i.e. nonsolvent) for the drug of interest, minocycline and PLGA had to be utilized. A solvent that does not dissolve minocycline well, will be not as likely to extract drug into the collection media as the microparticles set/precipitate. A solvent that is not good for the polymer will also help to precipitate the PLGA faster, resulting in better entrapment of the drug in the particles. As shown in our previous work (Chew et al., 2016), the ability of a solvent to better precipitate PLGA may result in the increase in drug entrapment efficiency in a PLGA scaffold. Furthermore, according to Zhang et al. (2019), it is important that the compatibility between the collection media and solvent used for electrospraying is higher than between the polymer and solvent system. This is key for the effective removal of the residual solvent in the particles into the collection media through diffusion due to a concentration gradient. Both DCM and methanol (electrospraying solvents) are compatible and miscible with isopropanol (i.e., the collection media). A low surface tension collection solvent surface is also important to ensure prevention of aggregation of particles and formation of spherical shaped microspheres (Zhang et al., 2019). The particles' shape changes from spherical to flat if the surface tension of the collection liquid increases.

The amount of drug used for fabrication and collection were investigated (Table 3). As expected, it was determined that when more drug was used in the fabrication, the drug loading (%) increased as more drug was present in the polymer/drug solution. The loading efficiency was not affected by the amount of drug used for fabrication. Overall, a higher amount of drug loading and loading efficiency was achieved when the evaporation collection method was used compared to the decant collection method. When the collection medium is decanted or removed and then the microparticles were allowed to dry, drug that was not encapsulated in the microparticles was lost with the decant solution. However, when the solution is only allowed to dry, the drug that was not encapsulated inside the particles, can still be present on the surface of the particles, resulting in the higher drug loading and loading efficiency.

Although associated with advantages, the liquid collection method also has its disadvantages including the loss of drug through diffusion into the collection media (Zhang et al., 2019). Although a suitable nonsolvent for PLGA and minocycline was used as the collection media, the loss of the drug into the isopropanol still occurred. Compared to the flat plate collection method used in our previous study (Rodriguez de Anda et al., 2019) where we were able to obtain high loading efficiencies (i.e., in the range: 71%–82%), the loading efficiencies obtained in this study were between 32% and 52%. The actual loading efficiency may be even lower as a lot of the drug, as indicated by the burst release in the first hour, may be on the surface of the particles. Zhang et al. (2019, 2021) incorporated polyethylene glycol (PEG) which a is a hydrophilic material to result in an increase in monodispersity and encapsulation efficiency of their minocycline-loaded microparticles. PLGA containing methyl and methylene hydrophobic groups is not conducive to mixing and encapsulating the hydrophilic drug minocycline. By incorporating PEG, the microspheres become more hydrophilic. However, it has also been shown that PEG can decrease encapsulation efficiency of water-soluble drugs with the increase in PEG content and MW as the PEG can occupy volume in the particles (L. Shi et al., 2021). However, the addition of PEG can results in better drug bioactivity as the hydrophilic additive can create pores in the hydrophobic polymer matrix to ease the release of acidic degradation products but in turn can also result in higher drug release (Bock et al., 2014). Thus, PEG can be possibly used to enhance the release of drugs that are hydrophobic from PLGA particles (Huang et al., 2013), however, may results in a high release for hydrophilic drugs.

The electrosprayed microparticles that were obtained ranged between 62 and 88 μm in diameter and were spherical shaped. Although there are advantages associated with nano-sized particles, such as the ability to cross biological barriers and ease in entering cells (Kohane, 2007), these particles are associated with limitations as well. Microparticles that are injected into a site of interested have a higher tendency to stay in place, and thus, preventing infiltration of particles in nontarget tissues and increasing bioavailability of the drug at the site of interest (Kohane et al., 2002, 2006). The surface area to volume ratio of a particle is inversely proportional to its radius and thus, smaller particles have higher surface area to volume ratio which can result in greater exposure to the external aqueous phase and increase water penetration which can lead to increased hydrolytic

degradation of the particles (Kohane, 2007). Thus, nanoparticles, compared to microparticles, will result in a higher loss of drug, increased burst release and more rapid release kinetics. Furthermore, small particles tend to aggregate more compared to larger particles (Kohane, 2007) thus, microparticles are associated with several advantages compared to nanoparticles and may be promising drug delivery systems for certain applications.

FTIR analysis confirmed that minocycline was successfully loaded into and/or on the surface of the microparticles as both PLGA and minocycline FTIR peaks were observed for the fabricated particles. The absence of additional FTIR peaks in both the minocycline-loaded PLGA microparticles and the residue obtained after evaporation indicates that the interaction between PLGA and minocycline was due to physical blending rather than chemical conjugation (Zhang et al., 2021). When the evaporation collection method was utilized, the size of the particles increased significantly. This could have resulted from increase in drug left in the collection media absorbing on the particles, resulting in a drug coat on the particles' surface. The spherical shaped particles had smaller spherical shaped particles on them. To further investigate what these smaller particles were, SEM imaging was performed on minocycline drug, minocycline dissolved in isopropanol and then allowing the isopropanol to evaporate, and by imaging what was left from the decant solution (Figure 3). Although minocycline drug powder had an elongated cylindrical shape before dissolving in isopropanol, once it was dissolved in isopropanol and then allowed to evaporate, the minocycline formed a crystalline structure that coated the glass petri dish. However, powder collected from allowing the isopropanol from the decant solution to evaporate, resulted in a similar small spherical shaped structure seem on the fabricated minocycline-loaded PLGA microparticles (i.e., larger spherical shaped structure). FTIR analysis revealed that the powder collected from the decant solution contained both PLGA and minocycline with stronger minocycline compared to PLGA associated peaks than the electrosprayed minocycline-loaded microparticles, indicating that the small spherical structure on the microparticles could be made of PLGA and minocycline, but a higher amount of minocycline compared to the particles. This suggests that the small spheres could be smaller size microparticles and they are further coated with excess minocycline that was not entrapped within the larger particles and left in the isopropanol collection media.

The release study demonstrated that a high amount of drug (i.e., %) was released within the first hour. Minocycline, compared to other tetracycline, contains two amino groups which results in its high solubility in water (Holmkvist et al., 2016). This therefore leads to fast release rates as the hydrophilic drug readily dissolve into the aqueous environment once in contact with it (Holmkvist et al., 2016). The burst release could also have resulted from a lot of the drug being on the surface of the particles that was adsorbed onto the particles from the collection media after evaporation of the collection solvent. This drug that is not encapsulated, can be easily released from the microparticles. Furthermore, drug on the surface could have also resulted from the electrospraying process. As seen by Zhang et al. (2021) through laser confocal images, the amount of minocycline on the microparticles surfaces increases with an increase in drug loading. The increase in drug concentration could resulted in the dissolved drug being unable to draw toward the center of the droplet as the solvent evaporated, and thus, remained at the droplet or particle surface, forming a layer of solute shell (Hong et al., 2008). To decrease the burst release from the microparticles, the microparticles can be incorporated into a scaffold or depot. Zhang et al. (2021) demonstrated that the incorporation of their minocycline-loaded microspheres into sucrose acetate isobutyrate (SAIB) depots can help curb the burst release seen with the microspheres alone as only a small amount of the drug on the surface may be released into the SAIB depots. This prevention of the burst release by incorporating the microparticles into SAIB depots was also seen in other studies (Lee et al., 2006; Wang et al., 2016; Yang et al., 2019). The release of minocycline directly from SAIB depots, without the microspheres component also resulted in a burst release thus, demonstrating the benefits of utilizing the combination of microparticles and a scaffold or depot instead of only using each of these components alone in the development of biomaterial systems which can sustain the release of drug while preventing a burst release.

The efficacy of the minocycline-loaded PLGA microparticles was tested through a cell viability assay against U87-MG glioblastoma cells with three other groups: the control, free drug, and blank microparticles. The minocycline-loaded PLGA microparticles resulted in significantly higher cell death compared to the control, free drug, and blank MP at all the drug concentrations tested. This indicates that loading the drug in the PLGA MP can help protect the drug from degradation and result in more efficient killing of the glioblastoma cells compared to the delivery of the drug by itself. The blank MP was not significantly different than the control, indicating that the PLGA MP are biocompatible. Liu et al. (2011, 2013) demonstrated that approximately 30 and 75 μM of minocycline is needed to achieve 80% and 50% cell survival, respectively. In this work, we investigated much higher concentration of minocycline (i.e., 200–400 μM) and observed around 80% cell survival at 400 μM. This difference in observation with Liu et al. (2011, 2013) could have resulted from the difference in cell density that was used for the MTT cell viability assays. In this work, we used a cell density of five times that used by Liu et al. (2011, 2013) (1×10^4 vs. 2×10^3 cells/well), and thus, a much higher concentration is needed to have an effect on the cells. The evaluation of the effects of the minocycline-loaded PLGA microparticles on normal cells (such as glia cell lines and primary astrocytes) versus neoplastic/glioma cell lines will be evaluated in the future.

5 | CONCLUSIONS

The electrospraying method of fabricating PLGA microparticles resulted in particles with higher loading efficiency compared to the emulsion solvent evaporation method and resulted in ease in obtaining the particles as tedious separation of the particles from the aqueous phase is not needed as in the emulsion solvent evaporation method (Xie et al., 2006). It was determined that

DCM:methanol was the optimal solvent combination for minocycline and a higher amount of methanol (i.e., 70:30) can result in a higher drug loading. All three solvent ratios investigated produced microparticles that were both spherical and smooth, with no pores on their surface. The hydrophilic nature of minocycline presented a challenge in encapsulating a high amount of drug and resulted in a burst release of the drug, however, the incorporation of the microparticles in a scaffold or depot in the future can help minimize the burst release and prolong the sustained release of the drug. The electrosprayed minocycline-loaded microparticles were able to elicit a significantly higher cytotoxic response in U-87 MG glioblastoma cells compared to minocycline delivered by itself and thus, suggesting the possibility of the microparticle to protect the bioactivity of the drug. In conclusion, electrospraying is a promising method to fabricate minocycline-loaded PLGA microparticles with high drug loading and loading efficiency compared to the emulsion solvent evaporation method but further optimization of the fabrication parameters needs to be investigated to increase the drug loading and sustained delivery of the drug.

AUTHOR CONTRIBUTIONS

Marco A. Arriaga, Juan A. Amieva Jr., Jaqueline Quintanilla, Angela Jimenez, Julio Ledezma, Silverio Lopez, Karen S. Martirosyan and Sue Anne Chew: contributed to the collection of data. **Marco A. Arriaga, Karen S. Martirosyan and Sue Anne Chew**: conceived the project and directed the project. **Marco A. Arriaga, Jaqueline Quintanilla, Karen S. Martirosyan and Sue Anne Chew**: finalized the writing of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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