Preparation of High-Payload, Prolonged-Release Biodegradable Poly(lactic-co-glycolic acid)-Based Tacrolimus Microspheres Using the Single-Jet Electrospray Method

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Tacrolimus-loaded poly(lactic-co-glycolic acid) microspheres (TAC-PLGA-M) can be administered for the long-term survival of transplanted organs due to their immunosuppressive activity. The purpose of our study was to optimize the parameters of the electrospray method, and to prepare TAC-PLGA-M with a high payload and desirable release properties. TAC-PLGA-M were prepared using the electrospray method. In vitro characterization and evaluation were performed using scanning electron microscopy, X-ray diffraction (XRD), differential scanning calorimetry (DSC), and Fourier-transform infrared spectroscopy. Drug-loading efficiency was greater than 80% in all formulations with a maximum loading capacity of 16.81±0.37%. XRD and DSC studies suggested that the drug was incorporated in an amorphous state or was molecularly dispersed in the microspheres. The in vitro release study showed prolonged release patterns. TAC-PLGA-M with enhanced drug loading and prolonged-release patterns were successfully prepared using the electrospray method.

Key words electrospray; microsphere; poly(lactic-co-glycolic acid); prolonged release; tacrolimus

Tacrolimus (TAC), a potent immunosuppressant drug, is primarily used to increase the survival time of transplanted organs.¹ The immunosuppressive activity of tacrolimus is mediated through the inhibition of calcineurin, which is a protein phosphatase found in the cytoplasm of T-cells, and the subsequent blockage of interleukin-2 production, leading to a decrease in T cell proliferation.² In clinical practice, tacrolimus is administered as a twice-daily dosing regimen. Modified-release tacrolimus with once daily administration has also been used safely.³ There are no reports regarding the use of prolonged release formulations of tacrolimus in clinical practice, although some polymer-based formulations for long-term delivery of tacrolimus have been used in animal model studies. As has been previously reported, a single dose of the new type of tacrolimus with biodegradable microspheres could keep the plasma concentration steady for two weeks, suggesting that immunosuppressive activity could be achieved with the use of polymer-based formulations.⁴,⁵ Subcutaneous injection of tacrolimus pellets, which could give a plasma concentration within the desired therapeutic window, provided effective immunosuppression for more than three months in rat model studies.⁶ Likewise, tacrolimus-loaded biodegradable microspheres reportedly achieved sustained release over a long period, giving flat parallel concentration profiles for 10d from the first day after a single subcutaneous administration in liver-transplanted rats.⁷

Long-term controlled delivery of drugs has been accomplished by the use of biodegradable polymeric particulate systems.⁸ Poly(lactic-co-glycolic acid) (PLGA) is a biodegradable polymer that undergoes hydrolysis in the body to produce two monomers: lactic acid and glycolic acid. The rate of degradation of PLGA and the release profile of drugs from PLGA-based formulations depend on the molecular weight of the polymer as well as the lactide to glycolide ratio.⁹ The mechanism of drug release from PLGA micro-particles is not fully understood, partly due to the complexity of the processes and the interactions present in such systems as well as the lack of understanding of the relationship between particle characteristics and drug release.

Electrospraying is an attractive method for preparation of microparticles that are suitable for drug delivery systems.¹⁰⁻¹³ It is a single-step process in which the target drug and polymer are dissolved in organic solvent, preferably with low boiling point, and the solution is sprayed using standard syringe at predetermined conditions of solute concentration, flow rate, voltage, and distance from the needle to the collector. The basic principle underlying electrospraying is the use of electrostatic forces to control the breaking of a liquid flow to form droplets, hence forming microparticles. Unlike single jet electrospraying, which uses a single nozzle for spraying of particles, multiple jet electrospray technique involves the use of multiple nozzles which can produce many spray jets simultaneously, and hence, can be used for large scale production of particles. Moreover, coaxial type of electrospraying technique can be used to produce multilayered microparticles and nanoparticles by introducing coaxial electrified jets.¹⁴ The principle underlying coaxial technology is that two immiscible liquids are injected separately from two concentrically located nozzles with one on the inside and the other on the outside.¹⁵ Several techniques have been used to prepare polymeric nano and microparticles, nonetheless, electrospraying has several advantages over conventional preparation methods that have

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led to an increased interest in the use of the electrospaying method for the fabrication of polymeric microparticles for pharmaceutical purpose. As reported previously, by virtue of the simplicity of the process and its suitability to overcome the limitations of other techniques, electrospaying can produce microparticles with controlled size and morphology without the use of surfactants at room temperature.\(^{17}\) Various agents such as simvastatin, celecoxib, budesonide, carbamazepine, bovine serum albumin, tamoxifen, naproxen have been encapsulated successfully with various carriers by the use of electrospaying technique.\(^ {18–24}\)

Traditional microparticle fabrication methods provide inhomogeneous release profiles and suffer a lack of batch-to-batch reproducibility, hindering their potential to up-scale and their translation into clinical practice. Electrospaying is a promising technique for generating reproducible particles of polycaprolactone. Since no toxic residue was detected by this process based on preliminary cell work using DNA quantification assay, the electrospaying method was validated as suitable for further loading of bioactive components.\(^ {25}\)

The purpose of our study was to optimize the parameters of electrospaying and preparation of Tacrolimus-loaded poly(lactic-co-glycolic acid) microspheres (TAC-PLGA-M) using a reproducible electrospaying method to provide particles with a narrow size distribution and desirable release patterns. Furthermore, we also aimed to maximize the loading efficiency and to augment the loading capacity of our system compared to previously reported methods. TAC-PLGA-M could be a potential delivery system for long-term immune suppression in organ transplantation.

### Experimental

#### Materials
Poly(lactic-co-glycolic acid) (50:50 d,l-PLGA with acid end group, molecular weight: 54 kDa) was purchased from Evonik Industries AG (Darmstadt, Germany), and tacrolimus was a generous gift from Hanmi Pharma Co., Ltd. (Seoul, Republic of Korea). Reagent-grade methylene chloride was purchased from Junsei Chemicals Co., Ltd. (Tokyo, Japan).

#### Preparation of TAC-PLGA-M
TAC-PLGA-M was prepared using a single-nozzle electrospaying machine (Fig. 1). The spraying system consisted of a voltage power source from NanoNC (Seoul, Republic of Korea) with a high voltage output, a high-precision mechanical syringe pump with adjustable flow rate from KD Scientific Inc. (Holliston, MA, U.S.A.), and a custom-built concentric stainless steel nozzle with outer and inner diameters of 0.80 and 0.55 mm, respectively. A jet was formed at the nozzle tip by adjusting electric voltage, flow rate, solute concentration in the organic solvent, and collection distance from the tip of the syringe pump to an aluminum sheet used as a collecting plate.

### Optimization of Formulation Parameters
The formulation variables in the experiment were concentration of solute, flow rate, voltage and collection distance. For the optimization of the total solute concentration, 2.5, 5, 7.5, 10, and 12.5% (w/v) of PLGA were prepared in methylene chloride and were sprayed at fixed flow rate of 0.5 mL/h, voltage of 15 kV and collection distance of 20 cm. Similarly, other variables were optimized as shown in Table 1, where the conditions for the preparation were optimized based on morphological examination of microspheres under scanning electron microscope (SEM).

Tacrolimus loaded PLGA microspheres were prepared with 7.5% (w/v) of total solute concentration in methylene chloride. Briefly, different weight ratios of tacrolimus and PLGA were dissolved in methylene chloride and sprayed by using electrospaying machine as described previously.

#### Characterization of Formulations
Scanning Electron Microscopy (SEM)

Morphological observations were made by scanning electron microscope (S-4100, Hitachi, Japan). To prepare the sample for microscopy, the microspheres were fixed on a brass stub using double-sided adhesive tape and vacuum-coated with platinum for 120 s using Ion Sputter (E-1030, Hitachi, Japan).

### Encapsulation Efficiency and Drug-Loading Capacity (LC)
To determine the encapsulation efficiency and drug-LC,

#### Table 1. Optimization of Variables with Blank PLGA Microspheres

| Formulations | PLGA (%) | Flow rate (mL/h) | Voltage (kV) | Collection distance (cm) |
|--------------|----------|-----------------|--------------|--------------------------|
| F1           | 2.5      | 0.5             | 15           | 20                       |
| F2           | 5        | 0.5             | 15           | 20                       |
| F3           | 7.5      | 0.5             | 15           | 20                       |
| F4           | 10       | 0.5             | 15           | 20                       |
| F5           | 12.5     | 0.5             | 15           | 20                       |
| F6           | 7.5      | 0.3             | 15           | 20                       |
| F7           | 7.5      | 0.7             | 15           | 20                       |
| F8           | 7.5      | 0.5             | 12           | 20                       |
| F9           | 7.5      | 0.5             | 18           | 20                       |
| F10          | 7.5      | 0.5             | 15           | 25                       |

Concentration of PLGA, flow rate, voltage, and collection distance were optimized. The conditions in F3 were considered optimized based on morphological observation of microspheres using scanning electron microscopy (SEM).
known amounts of formulations, theoretically equivalent to 1 mg of drug, were taken. Acetonitrile (1 mL) was then added to the tube containing microspheres and vortex-mixed to completely dissolve the formulations in acetonitrile for 3 h. The solutions were then filtered using 0.5-μm filter, diluted adequately, and analyzed by HPLC. The mobile phase consisted of acetonitrile and 0.1% phosphoric acid in a ratio of 70:30. Inertisol (4.6×150 mm; 5 μm) column was used as an analytical column. Flow rate of 1 mL/min and detection wavelength of 210 nm were used during analysis. The column temperature was maintained at 40°C. Encapsulation efficiency (EE) and drug-LC were calculated with the following formula as described previously:26–28

\[
EE(\%) = \frac{\text{Observed amount of TAC}}{\text{Added amount of TAC}} \times 100\%
\]

\[
\text{LC}(\%) = \frac{\text{Weight of TAC in microspheres}}{\text{Weight of microspheres}} \times 100\%
\]

X-Ray Diffraction (XRD)

To investigate the physical state of the drug and the formulations, XRD patterns were recorded using X-ray diffractometer (X’Pert MPD diffractometer, PANalytical, Almelo, the Netherlands) with a copper anode operated at a voltage of 40 kV and current of 30 mA. The diffraction patterns were obtained with an angular increment of 0.04°/s in the region of 10° ≤ 2θ ≤ 60° at ambient temperature.

Differential Scanning Calorimetry (DSC)

To identify the thermal behavior of the drug and the formulations, DSC (DSC-Q200, TA Instruments, U.S.A.) was used. The drug and formulations were separately heated with linear increase in temperature at a rate of 20°C/min and scanned from 40 to 180°C. An empty aluminum pan, which had a well-defined heat capacity over the range of temperatures scanned, was used as a reference.

Fourier Transform Infrared (FT-IR) Spectroscopy

To investigate the possible interaction between the drug and polymer, FT-IR spectroscopy was performed by attenuated total reflectance (ATR) method using Nicolet Nexus 670 FT-IR Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). FT-IR scanning was done from wave number 550–4000 cm⁻¹ at a resolution of 16.

In Vitro Drug Release Study

The in vitro drug release pattern of tacrolimus from the formulations was assessed in phosphate buffered saline (PBS, pH 7.4) at 37°C in a water bath by dialysis method, as previously reported.29,30 Tween 20 (1%) was used to increase the solubility of tacrolimus in the release medium. Briefly, weight equivalent of different formulations of TAC-PLGA-M containing 1 mg TAC were taken and a suspension was made with small volumes of the release medium. The suspension was then loaded into a dialysis membrane (MWCO 3.5 kDa), which was clamped at both ends to ensure no leakage took place. Then the dialysis membrane was kept gently in a 50-mL tube containing 10 mL of release medium. At predetermined time intervals, the entire release medium was taken out for analysis and replaced with an equivalent amount of fresh medium each time. HPLC analysis was performed as described previously.

Drug Release Kinetics: Model-Dependent Methods

In order to investigate the kinetics of drug release from the formulations, the drug release profiles were fitted to different model equations: zero-order (\(Q_t = Q_0 - K_t t\), where ‘Q’ is the amount of drug dissolved in time ‘t’; ‘Q_0’ is the initial amount of drug in the solution, and ‘K_t’ is the zero order rate constant), first order (\(\ln C = \ln C_0 - K' t\), where ‘C_0’ is the initial concentration of drug, ‘C’ is the drug remaining, ‘K’ is the first order rate constant, and ‘t’ is time), Higuchi (\(Q = K_H t^{1/2}\), where ‘Q’ is the amount of drug released, ‘t’ is the time, and ‘K_H’ is the Higuchi dissolution constant), Korsmeyer–Peppas (\(M_t/M_\infty = K \cdot t^{n}\), where ‘M_t/M_\infty’ is the fraction of drug released after time ‘t’, ‘K’ is the release constant and ‘n’ is the release exponent, which characterizes the different release mechanisms), and Hickson–Crowell (\(W_t/W_\infty^{1/3} = K t\), where ‘W_t’ is the initial amount of drug, ‘W_\infty’ is the remaining amount, ‘k’ is the constant and ‘t’ is time).31 The correlation coefficient ‘r²’ was used as an indicator for the best fit to the models.

Cytotoxicity Assay

Cytotoxicity was assessed by taking INS-1 cells as a model cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously, with some modifications. Briefly, 1×10⁴ INS-1 cells were seeded into 96-well plates; after 24-h incubation, the cells were exposed to different concentrations of blank polymeric microspheres for 48 h. MTT solution

![Fig. 2. Scanning Electron Microscopy (SEM) of Blank PLGA Microspheres in Various Conditions of Solute Concentration, Flow Rate, Voltage and Collection Distance](image)

The details of the conditions are given in Table 1. F3 was taken as the optimal condition to form monodispersed microspheres as revealed by SEM. Scale bar: 10 μm.
(1.25 mg mL⁻¹) was then added to each well and incubated for 3 h in the dark. Formazan crystals were dissolved by adding 100 µL of dimethyl sulfoxide (DMSO). Absorbance was then measured at 570 nm using a microplate reader (Multiskan EX, Thermo Scientific, Waltham, MA, U.S.A.).

Results and Discussion

Optimization of TAC-PLGA-M The optimized formulation was selected on the basis of surface topography and particle size distribution (Fig. 2). When observed under scanning electron microscope, homogeneous-sized microspheres could be observed at solute concentration 7.5% (w/v), flow rate 0.5 mL/h, voltage 15 kV, and collection distance 20 cm. At lower concentrations of solute, very irregularly shaped particles were observed, whereas at higher concentrations of solute, numerous fibers were observed. As previously reported, when the concentration of PLGA was increased, the mean distance among the macromolecules was reduced. As a result, fibers were more likely to be produced due to improvement of intermolecular entanglements. Our results were consistent with previous findings, such that the polymer concentration was the most pivotal factor determining the topography of particles. Decreasing the flow rate produced particles with more widely dispersed size ranges. High flow rate resulted in close intermolecular entanglements. As a result, numerous fibers were observed when the collection distance was increased to 25 cm.

TAC-loaded formulations prepared at the optimized conditions, as shown in Table 2, revealed rough surfaces by virtue of rapid evaporation of methylene chloride during the process (Fig. 3). Particles, upon SEM observation, ranged from 3 to 6 µm. Some smaller particles could be observed because the droplets generated by electrospraying are likely to undergo Coulomb fission during evaporation, and hence, there is a chance of producing offspring droplets. The fission of particles occurs due to shrinkage during evaporation without losing charge, which leads to attainment of Rayleigh limit.

Encapsulation Efficiency and Drug-LC The effects of TAC and PLGA ratio on encapsulation efficiency and drug-LC of TAC-PLGA-M were investigated. The encapsulation efficiency and drug-LC in different loading conditions are shown in Table 3. Encapsulation efficiency ranged from a minimum of 84.03 ± 1.83% to a maximum of 90.77 ± 0.95%. By virtue of the high encapsulation efficiency, less amount of drug is lost with electrospraying compared with other methods, a definite advantage of electrospraying. Although the encapsulation efficiency appeared to decrease as the ratio of drug to polymer increased, the LC (16.81 ± 0.37%) was superior to other reported methods.

X-Ray Diffraction Pattern The X-ray diffraction pattern of TAC revealed characteristic peaks of the drug, most prominently at 2θ value of 10–25°, which indicates high crystalline nature of the drug. The disappearance of these characteristic peaks in the drug-loaded formulations suggests that the drug was molecularly dispersed or was present in amorphous state within the microspheres (Fig. 4).

Differential Scanning Calorimetry (DSC) DSC thermogram of TAC exhibited a sharp endothermic peak around 140°C, which indicates the melting point of the drug and assures that the drug was present in crystalline form. However, no endothermic peak of drug was seen in the formulations (Fig. 5), which further suggests that the drug was molecularly dispersed or was present in amorphous form within the microspheres.

FT-IR Spectrometry As illustrated in Fig. 6, the characteristic bands of TAC were observed in FT-IR, viz. O–H stretching vibration at 3450 cm⁻¹, C=O stretching vibrations at 1733 and 1690 cm⁻¹, C–O (ester) stretching vibration at 1733 and 1690 cm⁻¹, C–O (ester) stretching vibration at

![Fig. 3. Scanning Electron Microscopy (SEM) of TAC-PLGA-M in Various Weight Ratios of TAC to PLGA](image_url)

(a) TAC : PLGA = 10 : 90, (b) TAC : PLGA = 15 : 85, and (c) TAC : PLGA = 20 : 80. The concentration of total solute was kept constant in all formulations. Scale bar: 10 µm.

### Table 2. Preparation of TAC-PLGA-M with Different Ratios of the Drug and Polymer

| Formulations | TAC : PLGA (wt. ratio) | Volume of methylene chloride (mL) |
|--------------|------------------------|----------------------------------|
| a            | 10 : 90                | 2                                |
| b            | 15 : 85                | 2                                |
| c            | 20 : 80                | 2                                |

(a) TAC : PLGA = 10 : 90, (b) TAC : PLGA = 15 : 85, and (c) TAC : PLGA = 20 : 80. TAC and PLGA were dissolved in 2 mL of methylene chloride and the solution was subjected to electrospray under optimized conditions at room temperature.

### Table 3. Encapsulation Efficiency and Loading Capacity of TAC-PLGA-M at Various Ratios of TAC and PLGA

| Formulations | TAC : PLGA (wt. ratio) | EE (%) | LC (%) |
|--------------|------------------------|--------|--------|
| a            | 10 : 90                | 90.77±0.95 | 9.41±0.41 |
| b            | 15 : 85                | 87.99±3.72 | 13.39±0.27 |
| c            | 20 : 80                | 84.03±1.83 | 16.81±0.37 |

EE: Encapsulation efficiency, LC: drug loading capacity. The values of EE and LC represent the mean±S.D. (n=3). The compositions were taken at various weight ratios of TAC to PLGA.
The majority of the peaks overlapped, weakened, shifted or disappeared in the drug-loaded formulations, probably due to hydrogen bonds between the polymer and the drug. The appearance of no new peaks in the formulations indicates that there is no chemical interaction between the polymer and the drug. Similar results were observed in previous works, such that the majority of the drug peaks were not observed by virtue of hydrogen bonds from dissolving tacrolimus in self-microemulsifying drug delivery system (SMEDDS) excipients. 38) In Vitro Release Study The release study on the formulations revealed prolonged release profiles of TAC, extending to more than 20d. As reported previously, PLGA microspheres loaded with risperidone showed a sustained release profile for

![Fig. 4. X-Ray Diffraction (XRD) Patterns of TAC and TAC-PLGA-M in Various Weight Ratios of TAC to PLGA](image)

(a) TAC free drug, (b) TAC:PLGA=20:80, (c) TAC:PLGA=15:85, and (d) TAC:PLGA=10:90. In contrast to the free drug, no characteristic peaks of drug were observed in the patterns of drug-loaded formulations.

![Fig. 5. Differential Scanning Calorimetry (DSC) Thermograms of TAC and TAC-PLGA-M in Various Weight Ratios of TAC to PLGA](image)

(a) TAC free drug, (b) TAC:PLGA=20:80, (c) TAC:PLGA=15:85, and (d) TAC:PLGA=10:90. In contrast to the free drug, no characteristic endothermic peak of the drug was seen in the curves of formulations.

![Fig. 6. Fourier Transform Infrared (FT-IR) Spectroscopy of TAC, PLGA, and TAC-PLGA-M in Various Weight Ratios of TAC to PLGA](image)

(a) TAC free drug, (b) PLGA, (c) TAC:PLGA=20:80, (d) TAC:PLGA=15:85, and (e) TAC:PLGA=10:90.

![Fig. 7. In-Vitro Release Profiles of TAC-PLGA-M](image)

(a) TAC:PLGA=20:80, (b) TAC:PLGA=15:85, and (c) TAC:PLGA=10:90.

The release study was performed in phosphate buffered saline, pH 7.4, with 1% Tween 20. Whole media was replaced every other day after sampling to maintain sink conditions in the release media. The values represent the mean±S.D. (n=3). The cumulative release values were fitted to different kinetics equations. [Zero order equation: \( Q_o - Q_t = K_0 t \), first order (\( \ln C = \ln C_0 - K t \)), Higuchi (\( Q = K H \cdot t^{1/2} \)), Korsmeyer–Peppas (\( M_t/M_\infty = K \cdot t^n \)), and Hickson–Crowell (\( W_0^{1/3} - W_t^{1/3} = \kappa t \)).]

| Formulation | TAC:PLGA (wt. ratio) | Zero order \((r^2)\) | First order \((r^2)\) | Korsmeyer–Peppas \((r^2)\) | Hickson–Crowell \((r^2)\) | Higuchi \((r^2)\) |
|-------------|---------------------|-----------------|-----------------|-------------------|-------------------|------------------|
| a           | 10:90               | 0.9845          | 0.9074          | 0.9665            | 0.9511            | 0.7066           |
| b           | 15:85               | 0.9821          | 0.9347          | 0.9425            | 0.9622            | 0.8058           |
| c           | 20:80               | 0.9489          | 0.9045          | 0.9407            | 0.9316            | 0.7601           |

The value of \( r^2 \) was considered to determine the release order kinetics. In the Korsmeyer–Peppas model, ‘n’ explains the mechanism of drug release. The values of ‘n’ were (a) 0.835, (b) 0.685, and (c) 0.738. All the values ranged between 0.5–1.0, which refer to a release mechanism of non-Fickian diffusion or anomalous transport.
4 weeks as measured from the plasma drug concentration suggesting a good potential of PLGA microspheres for sustained release delivery system.\(^\text{39}\) As the loading amount of tacrolimus was increased, the amount of PLGA per unit of the drug loaded was also decreased. As a result, the relative abundance of PLGA in the microspheres was decreased with increase in loading, which results in increase in the porosity of the microspheres with increase in drug loading. This ultimately gave the faster release patterns as the amount of drug loaded was increased (Fig. 7). This suggests that along with the molecular weight and composition of PLGA, the release rate is also controlled by the ratio of TAC to PLGA in the formulation.

A similar trend in the release of docetaxel from PLGA nanoparticles was found where an increase in the amount of drug loading showed a higher rate of release.\(^\text{40}\) As observed from the release profile, a significant initial burst release was absent, which indicates that TAC, being sufficiently hydrophobic, was well encapsulated inside the microspheres.

After fitting the release profile with different kinetics models of drug release as shown in Table 4, the coefficients of correlation \((r^2)\) were taken into consideration in order to investigate the release model. A high degree of correlation to zero order equation \((r^2\) value ranging from 0.9489 to 0.9845) indicates that drug release is independent of the concentration of loaded drug in the microspheres. This could be an advantage of our system for its application to clinical settings, as a known amount of drug can be released in a certain interval, avoiding toxic effects due to overdose. Moreover, the release profiles showed good correlation with Korsmeyer–Peppas model \((r^2\) value ranging from 0.9407 to 0.9665), indicating that diffusion is the main mechanism involved in the release of drug from the formulations. Furthermore, the values of ‘\(n\)’ in Korsmeyer–Peppas model were 0.835, 0.685, and 0.738 for formulations \(a\), \(b\), and \(c\), respectively. This indicates that the mechanism of drug release is related to diffusion of the drug as well as degradation of the polymer. Values \(0.5 < n < 1.0\) in Korsmeyer–Peppas model are referred to as anomalous transport or non-Fickian diffusion. The scanning electron microscopy of the TAC-PLGA microspheres after release study revealed amorphous-like transition, as observed in Fig. 8, suggesting that most of the microspheres were degraded during the release study. A very few microspheres with small

![SEM images of degraded TAC-PLGA microspheres](image)

**Fig. 8.** Scanning Electron Microscopy (SEM) of Degraded TAC-PLGA-M after 25d of Release

(a, b) TAC:PLGA=10:90 at magnification 3.00K and 15.0K, (c, d) TAC:PLGA=15:85 at magnification 3.00K and 15.0K, and (e, f) TAC:PLGA=20:80 at magnification 3.00K and 15.0K. The degraded microspheres were retrieved from the dialysis bag on the 25th day of release, dried and observed by SEM. Scale bar: 10\(\mu\)m (left panel) and 2\(\mu\)m (right panel).

![Cell viability graph](image)

**Fig. 9.** Cytotoxicity Assay (MTT) of Blank PLGA Microspheres in INS-1 Cell Line after Exposure of the Cells for 48h

The concentration of blank microspheres corresponded to the molar concentration of TAC present in the formulation containing TAC to PLGA ratio of 10:90. Each value represents the mean\(\pm\)S.D. \((n=3)\). \(p\) Values between control and each group was statistically non-significant [confidence interval: 95%].
cracks on the surface were observed at higher magnification, which further indicates the degradation of the polymer during release.

**Cytotoxicity Assay** The cytotoxicity of blank polymeric microspheres was assessed in the INS-1 cell line as illustrated in Fig. 9. The concentration of blank microspheres corresponded to the molar concentration of TAC present in the formulation containing drug to polymer ratio of 10:90, which contains the highest amount of PLGA among the formulations in equivalent weight of TAC. No significant cytotoxicity or adverse effect on proliferation was observed in comparison to control, which indicates that no residual traces of the organic solvent in toxic dose were present in the microspheres. Methylen chloride, which was used in our formulation, has some environmental and safety risks, so it needs to be completely removed from the microspheres to comply with the safety guidelines of The International Conference on Harmonization (ICH). Our results strongly indicate that the microspheres produced by our method could be used to deliver various drugs or biological agents in vivo without any significant cytotoxicity. Moreover, they can be safely used to co-deliver cells and drugs that can improve cell viability or functionality for improved cytomedicine therapy. However, the carrier system was non-toxic to insulin secreting cell line (INS-1 cells), it is obvious that the cytotoxicity has to be evaluated in other cells or cell lines if drugs loaded in to these particles are to be treated.

**Conclusion** The high drug encapsulation efficiency and drug-LC of TAC-PLGA-M (84.03 and 16.81%, respectively) and the lack of any significant amount of burst release in the initial days of *in-vitro* release indicated that the drug was well incorporated inside the microspheres. XRD and DSC analysis indicated that the drug was molecularly dispersed within the microspheres or was present in amorphous form. Prolonged release of TAC was observed for up to 20d, indicating a strong potential for use of the microspheres for long-term attainment of plasma therapeutic level of targeted drug. Thus, the TAC-loaded microspheres are a potential drug delivery system for long-term immune suppression without significant toxic effects in organ transplantations. The cytotoxicity assay also indicated that our delivery system could be a potential template to deliver various drugs as well as biological agents *in vivo* for long-term achievement of plasma therapeutic levels using subcutaneous route of drug delivery. However, *in vivo* pharmacokinetic studies need to be evaluated for application of the polymer-based TAC microspheres to clinical practice. Electrospaying could be an alternative preparation method for prolonged release drug delivery systems. Due to its reproducibility, electrospaying has the potential to be scaled up to industrial level for the preparation of various formulations that could be applied in clinical settings in the near future.

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**Conflict of Interest** The authors declare no conflict of interest.

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