Combined local delivery of tacrolimus and stem cells in hydrogel for enhancing peripheral nerve regeneration

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Author contributions

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Abstract

The application of scaffold-based stem cell transplantation to enhance peripheral nerve regeneration has great potential. Recently, the neuroregenerative potential of tacrolimus (an FDA-approved immunosuppressant) has been explored. In this study, a fibrin gel-based drug delivery system for sustained and localized tacrolimus release was combined with rat adipose-derived mesenchymal stem cells (MSC) to investigate cell viability *in vitro*. Tacrolimus was encapsulated in poly(lactic-co-glycolic) acid (PLGA) microspheres and suspended in fibrin hydrogel, using concentrations of 0.01 ng/mL and 100 ng/mL. Drug release over time was measured. MSCs were cultured in drug released media collected at various days to mimic systemic exposure. MSCs were combined with (i) hydrogel only, (ii) empty PLGA microspheres in hydrogel, (iii) 0.01 ng/mL and (iv) 100 ng/mL of tacrolimus PLGA microspheres in hydrogel. Stem cell presence and viability were evaluated. A sustained release of 100 ng/mL tacrolimus microspheres was observed up to 35 days. Stem cell presence was confirmed and cell viability was observed up to seven days, with no significant differences between groups. This study suggests that combined delivery of 100 ng/mL tacrolimus and MSCs in fibrin hydrogel does not result in cytotoxic effects and could be used to enhance peripheral nerve regeneration.

Keywords (3-5)

Nerve injury, tacrolimus, drug delivery, stem cells, tissue engineering

Introduction

Peripheral nerve injuries represent a substantial clinical problem with limited treatment options (Hussain et al., 2020). Following severe nerve trauma resulting in segmental defects, reconstruction with interpositional grafts or conduits is necessary to avoid excessive tension with primary end-to-end neurorraphy (Sunderland et al., 2004; Terzis, Faibisoff, & Williams, 1975). Options vary from the use of autologous or allogeneic nerve grafts to the use of synthetic or processed nerve graft alternatives (Ducic, Fu, & Iorio, 2012; Moore et al., 2011; Samadian et al., 2020). The application of processed nerve allografts has failed to consistently overcome nerve defects greater than 3 cm in length and their outcomes remain inferior to autografts (Rbia & Shin, 2017; Safa et al., 2020; Shin, 2014). While the decelluarization of nerve allografts reduces graft rejection and eliminates the need for systemic immunosuppression, the process also removes Schwann cells, destroys neurotrophic factors and alters the extracellular matrix resulting in reduced regenerative potency (Moore et al., 2011). Research has been dedicated to enhancing processed nerve allografts with cellular support, particularly stem cells, which may differentiate into various pro-regenerative and supportive cell types (Mathot, Shin, & Van Wijnen, 2019).

The outcome of scaffold-based stem cell transplantation in peripheral nerve injury remains unsatisfactory due to poor survival of the transplanted cells, most likely due to immune rejection (Yi et al., 2020). Systemic immunosuppression using tacrolimus (FK506), an U.S. Food and Drug Administration (FDA)-approved immunosuppressant, may overcome transplanted graft and cell rejection (Tung, 2015). However, systemic immunosuppression for transplantation causes various undesired side effects with long-term use, including increased risk of infection, drug toxicity resulting in hypertension and nephrotoxicity (Bulatova, Yousef, Al-Khayyat, & Qosa, 2011;

Saffari et al., 2019; Tung, 2010). Local and controlled delivery of immunosuppressant medications using various biomaterials has been demonstrating promising results, while minimizing systemic administration side effects (Davis et al., 2018; Kevin J. Zuo et al., 2020).

Recently, the neuroregenerative properties of tacrolimus have received more attention (Daneri-Becerra, Patino-Gaillez, & Galigniana, 2020; Saffari et al., 2019; Tajdaran, Chan, Shoichet, Gordon, & Borschel, 2019; Tajdaran, Chan, Zhang, Gordon, & Borschel, 2019; Zuo, Saffari, Chan, Shin, & Borschel, 2020). While it was first investigated for peripheral nerve regeneration via systemic administration, recent studies have been focusing on directing tacrolimus' release to the nerve repair site using localized delivery vehicles (Azizi, Mohammadi, Amini, & Fallah, 2012; Davis et al., 2018; Saffari et al., 2019; Unadkat et al., 2017). Another viable option to enhance the ability of processed nerve allografts to support axon regeneration is seeding with adipose-derived mesenchymal stem cells (MSC). MSCs stimulate secretion of neurotrophic and angiogenic factors which enhance peripheral nerve regeneration (Mathot, 2021; Rbia et al., 2019). Simultaneous delivery of tacrolimus and MSCs to a site of nerve injury may work synergistically to increase MSC survival by countering immune rejection and providing direct neuroregenerative support. Therefore, the purpose of this study was to determine the extent to which combined application of locally delivered tacrolimus and MSCs in a fibrin gel may enhance the viability of MSC.

Methods

Ethical consent for animal use in this study was approved by the Institutional Animal Care and Use Committee (IACUC, A4790-19).

Experimental design

According to a previously established method, tacrolimus was encapsulated in poly(lactic-co-glycolic acid) (PLGA) microspheres (Tajdaran, Shoichet, Gordon, & Borschel, 2015). In experiment A, tacrolimus microspheres were incubated in phosphate buffered saline (PBS) over a course of 35 days to measure release profiles. MSCs were incubated in collected supernatant PBS containing tacrolimus at 7, 15 and 28 days, to mimic systemic exposure to different concentrations and to evaluate MSC viability (Table 1). In experiment B, MSCs were combined with (i) hydrogel, (ii) empty PLGA microspheres in hydrogel, (iii) 0.01ng/mL tacrolimus PLGA microspheres in hydrogel and (iv) 100 ng/mL tacrolimus PLGA microspheres in hydrogel. MSC presence and viability were evaluated (Table 2). Low drug concentrations of 0.01 ng/mL and high drug concentrations of 100 ng/mL were previously found to optimize neurite outgrowth *in vitro* in a bimodal fashion, and these dose concentrations were therefore selected in this experiment (Tajdaran, Chan, Zhang, et al., 2019).

Microsphere synthesis

Tacrolimus was encapsulated in PLGA microspheres using a single emulsion solvent evaporation technique as described by Tajdaran and colleagues (Tajdaran et al., 2015). The organic phase consisted of dichloromethane (DCM) and acetone with a 75/25 ratio, 230 mg PLGA, 12.5 mg $MgCO_3$, and 12 mg tacrolimus. This was added to 25 mL of 2.5% polyvinyl alcohol (PVA) and 10% NaCl. The emulsion was formed via homogenization at 6,000 rpm for 60 seconds. Then, the solution was stirred into a 0.25% PVA and 10% NaCl hardening bath at 125 rpm for 3 hours. The resulting suspension solution was collected, washed by centrifugation, lyophilized, and kept frozen

at -20˚C until use. Three batches of microspheres were synthesized and characterized for these experiments.

Microsphere characterization

The mean ± standard deviation (SD) microsphere diameter was determined per batch with scanning electron microscopy. Samples were obtained and mounted onto stainless steel platforms and sputter coated with a 20 nm layer of gold with a Leica EM ACE200 sputter coated (Wetzlar, Germany). Images were taken using a FEI XL30 environmental scanning electron microscope (ESEM, Oregon, USA), with a beam energy of 20 kV. Approximately 100 microspheres were measured per batch using ImageJ and the pooled mean ± SD microsphere diameter was calculated to be 18.3 ± 4.2 μm.

The microspheres were further characterized to determine the mean ± SD encapsulation efficiency of tacrolimus in PLGA microspheres. Samples weighing 5 mg were taken from each batch and dissolved in 1 mL acetonitrile (ACN). The samples were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), according to previously described protocols (Tajdaran et al., 2015). Analyte peaks were measured and the encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulation efficiency} = \frac{mass_{tacrolimus\ detected}}{mass_{theoretical\ loading\ of\ tacrolimus}} \times 100\%$$

The pooled mean ± SD tacrolimus encapsulation efficiency in the PLGA microspheres was 47.1 ± 3.2 %. Mean ± SD loading of tacrolimus (μg tacrolimus/ mg microspheres) was derived from

the encapsulation efficiency per batch and used to determine the mass of microspheres needed to achieve a desired dose of tacrolimus.

Tacrolimus release profiles from fibrin gel

The release of tacrolimus from fibrin hydrogel was evaluated by incubating hydrogels containing 0.01 ng/mL and 100 ng/mL of tacrolimus microspheres in 1 mL PBS in a 37 °C incubator with 5% CO_2 on agitation. The PBS was collected and replenished over 35 days and analyzed for tacrolimus content in triplicate for both concentrations. Collected PBS was stored at -20°C until analysis.

Tacrolimus detection analysis

A Waters Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization (ESI) source (Milford, MA) was operated in positive electrospray ionization mode. The mass spectrometer was coupled to a Waters Acquity H class ultra-performance liquid chromatography system (Milford, MA). The separation of tacrolimus and internal standard (Cyclosporine A) was performed using an Agilent InfinityLab Poroshell 120 EC-C18 column, 2.1 x 100 mm, 2.7 µm (ChromTech, Apple Valley, MN), with a mobile phase (MP) composed of 10 mM Ammonium Acetate + 0.2% Formic acid in Water (MP A) and 10 mM Ammonium Acetate + 0.2% Formic acid in Methanol (MP B) operating at a flow rate of 0.3 mL/min and a sample injection volume of 10 µL. Column temperature was set at 40°C and autosampler temperature at 4°C. Drug elution consisted of a gradient starting at 30:70 (MP A:MP B, v/v) changing to 5:95 (MP A:MP B, v/v) from 0-4 minutes and then held until 6 minutes. The MP composition was then returned to initial conditions over 1 minute and then held there for 2 minutes. Data was acquired and analyzed with Waters MassLynx v4.1 software. Quantification was performed using multiple reactions

monitoring of the ammonium-adduct transition masses of tacrolimus (m/z 821.7→768.6) with a cone voltage of 18, collision voltage of 18 and internal standard Cyclosporine A (m/z 1203.1→100.0) with a cone voltage of 16 and collision voltage of 74. Data was acquired and analyzed with Waters MassLynx v4.1 software.

Mesenchymal stem cell preparation and culture

Rat MSCs from a previously characterized lineage were used for experiments (Rbia, Bulstra, Bishop, van Wijnen, & Shin, 2018). These MSCs were derived from the inguinal fat pad of inbred male Lewis rats according to a previously described protocol by Kingham and colleagues (Kingham et al., 2007). MSCs were characterized by plastic adherence, pluripotency towards mesodermal lineages, the expression of the MSC surface markers CD29 (88.2%) and CD90 (88.3%), and the absence of hematopoietic cell surface markers CD34 (91.1% absent) and CD45 (86.0% absent) (Mathot et al., 2020). Cells were stored in liquid nitrogen, thawed and the stromal cell pellet of passage 3 cells was re-suspended in growth medium consisting of advanced Minimum Essential Medium (α-MEM (1x); Life Technologies Corporation, NY, USA), 5% platelet lysate (PLTMax®; Mill Creek Life Sciences, MN, USA), 1% Penicillin/Streptomycin (Penicillin-Streptomycin (10.000 U/mL; Life Technologies Corporation, NY, USA), 1% GlutaMAX (GlutaMAX Supplement 100X; Life Technologies Corporation, NY, USA) and 0.2% Heparin (Heparin Sodium Injection, USP, 1.000 USP units per mL; Fresenius Kabi, IL, USA). Cultures were maintained at subconfluent levels in a 37 °C incubator with 5% CO_2 and passaged with TrypLE (Invitrogen, UK). Growth medium was changed every 72 hours. MSCs delivered in the gel were of passage 5.

Drug and stem cell composite delivery

Twenty-four-well cell culture plates were coated with pHEMA (poly 2-hydroxyethyl methacrylate; Sigma-Aldrich, MO, USA) to prevent migration of cells to the plastic surface of the well. For experiment A, 1 x 10^4 MSCs were incubated in 250 mL of collected supernatant PBS containing tacrolimus at 7, 15 and 28 days combined with 250 mL of growth medium per well in a 37 °C incubator with 5% CO_2. For experiment B, the fibrin hydrogel was prepared using a Tisseel® glue kit (Baxter Healthcare, IL). A total volume of 80 µL per gel was prepared by mixing equal parts of fibrinogen (67-106 mg/mL, 40 µL) and thrombin (40-63 IU/mL, 40 µL). MSCs at a concentration of 1 x 10^4 cells per 80 µL of gel were incorporated into the thrombin solution before mixing this with fibrinogen (Experiment B, group I). In group II, empty PLGA microspheres were mixed in fibrinogen and combined with 1 x 10^4 cells, incorporated in thrombin, to form 80 µL of gel. In group III and IV, tacrolimus microspheres were mixed with fibrinogen at concentrations of 0.01 ng/mL and 100 ng/mL. These microsphere suspensions were mixed with 1 x 10^4 MSCs, incorporated in thrombin, to create the hydrogel delivery system infused with stem cells and tacrolimus (HIST). Gels were incubated in 500 mL growth medium per well in a 37 °C incubator with 5% CO_2. Growth medium was changed every 72 hours.

Immunohistochemical staining against CD90

The expression of CD90, a canonical stem cell surface marker, was combined with the Live/Dead stain to confirm detection of stem cells (Saldanha-Araujo et al., 2012). After gels were fixed in formalin 10%, cell suspensions were blocked at room temperature (RT) for 1 hour (Purified Mouse Anti-Rat CD32, 1:1000; BD Pharmingen TM, CA, USA) to avoid unspecific binding. Samples were washed and stained against CD90 antibody (Monoclonal Mouse Anti-Rat 1:100, Bio-Rad,

Hercules, CA, USA) overnight at 4°C. The following day, cell supsensions consecutively underwent a sequence of washing steps and incubation with donkey anti-mouse Alexa Fluor 568 (1:500; Sigma) at RT for 2 hours. Samples were visualized using the confocal microscope (Zeiss LSM 780, Carl Zeiss Surgical GmbH, Oberkochen, Germany).

Stem cell viability

Live/Dead staining

The distribution and viability of MSCs in the gel was determined using LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen, Life Technologies Corporation, NY, USA). The kit was prepared according to its protocol, mixing 10 mL of sterile PBS, with 20 µL of Ethidium homodimer-1 and 10 µL of Calcein, in protection of light. Per well, 0.5 mL of this mixture was added and after 20 minutes this was rinsed with PBS and visualized using a confocal microscope. Live/Dead stainings were performed in triplicate for each group at 24 hours, 48 hours, 72 hours and 7 days after incubation.

Metabolic activity

To dermine the effect of the fibrin gel and tacrolimus on the viability of the MSCs and to compare the vulnerability of MSCs incubated in higher and direct incubation of tacrolimus, (3-(4,5-dimethylthiazol-2-yl) −5-(3-carboxymethoxyphenyl) −2-(4- sulfophenyl) −2H-tetrazolium) (MTS) assays (CellTiter 96® Aqueous One Cell Proliferation Assay, Promega, Madison, USA) were performed according to the manufacturer's instructions. This assay is a method for sensitive quantification of viable cells, assessing cell proliferation, cell viability and cytotoxicity (Cory, Owen, Barltrop, & Cory, 1991). Wells filled with PBS and MTS reagent only served as negative

control and for subtraction of the background signal. Wells plated with MSCs, served as positive control. MTS assays were performed in triplicate for each group at 48 hours after incubation. Results were analyzed using a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, USA) at an absorbance wavelength of 490 nm. The metabolic activity of MSCs in the vicinity of the fibrin gel and tacrolimus was expressed as a ratio of the metabolic activity of MSCs only.

Statistical analysis

Viability results were analyzed and compared to MSCs incubated in media (positive control). Analysis of variance (ANOVA) with Bonferroni post-hoc tests were used for comparisons between groups. Results were reported as the mean and standard error or the mean (SEM), and the level of significance was set at $\alpha \leq 0.05$.

Results

In vitro tacrolimus release from fibrin gel

Tacrolimus was encapsulated in microspheres to temporally control drug release. Microspheres containing 0.01 ng/mL tacrolimus showed a rapid increase up to a cumulative release of 99.9% by day 1 (mass released 87.59 ng, cumulative mass of 87.59 ng) which reached a 100% cumulative release by day 3 (mass released 44.24 ng, cumulative mass of 87.64 ng). The 0.01 ng/mL tacrolimus fibrin gel construct showed depletion of tacrolimus by 13 days (mass released 520 pg, cumulative mass of 87.64 ng, Supplementary Online Material Figure 1). The release profile of 100 ng/mL tacrolimus microspheres was found to be up to 35 days (Figure 1). This release occured in two distinct phases: from day 0-15 and day 15-35 (Figure 1A). The fibrin gel construct began

releasing 40 μg per day of tacrolimus which gradually increased up to a cumulative mass around 120 μg (mass released 4.94 μg) at day 13. A stepped cumulative increase was then observed from day 15 onwards (mass released 95.50 μg, cumulative mass of 215 μg) which further increased to a cumulative mass of 238 μg tacrolimus (mass released 5.81 μg) by day 35 (Figure 1B).

MSC incubation in collected PBS containing tacrolimus

Obtained Live/Dead micrographs of MSCs incubated in collected PBS containing 0.01 ng/mL and 100 ng/mL tacrolimus are shown in Figure 2. No differences in cell viability were observed when comparing culture in tacrolimus-containing PBS of day 7, 15 and 28 (Figure 3). Quantifying Live/Dead staining, cell viability ratios for cultures in 0.01 ng/mL tacrolimus containing PBS were found to be 99.2 ± 1.3% (mean±SEM), 93.9 ± 5.6% and 96.4 ± 1.8% for days 7, 15 and 28 respectively. For cultures incubated in 100 ng/mL of tacrolimus, cell viability ratios were 93.4±0.9%, 102.0±0.7% and 98.6±1.5% for day 7, 15 and 28, respectively (Figure 3A). The viability ratios using absorbance ratios (MTS assay) are shown in Figure 3B. Although a higher viability was found in tacrolimus-containing PBS of day 28, no significant differences were found. MTS results revealed cell viability ratios of 105.6±6.1%, 113.2±8.2% and 147.0±6.8% for 0.01ng/mL and 109.6±13.6%, 105.1±3.2% and 133.5±3.8% for 100 ng/mL in tacrolimus-containing PBS of days 7, 15 and 28, respectively.

Combined MSC and tacrolimus incubation in hydrogel

Microscopic CD90 and Live/Dead images of MSCs cultured in hydrogel groups are shown in Figure 4. CD90 confirmed stem cell presence in all hydrogel groups. Quantified Live/Dead and

MTS results found no significant differences between groups and time points, thus, cell viability was not reduced (Figure 5). Overall, Live/Dead results showed that the cell viability of all groups at 24 hours, 48 hours, 72 hours and 7 days was around 100%. Cell viability of the empty PLGA microspheres group at 7 days could not be quantified as a result of depleted gel (Figure 5A). MTS assay results found cell viability to be 73.6±1.3% when combined with hydrogel only, 77.2±5.1% when combined with PLGA empty microspheres, 64.6±3.5% for HIST 0.01 ng/mL and 65.4±1.1% for HIST 100 ng/mL, respectively (Figure 5B).

Discussion

Enhancing peripheral nerve repair and regeneration remains a challenge in tissue engineering and regenerative medicine (Hussain et al., 2020; Samadian et al., 2020). Properties of the local nerve injury environment have been recognized as an important factor of nerve regeneration and alteration of the local environment could enhance this process to potentially improve nerve repair outcomes (He et al., 2016; Saffari et al., 2020). Using a fibrin gel-based drug delivery system with sustained release would allow for the modulation of the environment over a prolonged time and overcome issues that are associated with non-degradable delivery methods such as nerve fibrosis and chronic nerve injury (Guenego et al., 2016; Guilhem et al., 2009; Merle, Dellon, Campbell, & Chang, 1989).

Nerve tissue engineering is becoming more prominent as artificial or naturally-derived biomaterials provide support for axonal growth and are carriers for nerve drug delivery applications at the same time (Belanger et al., 2016; Massoumi, Hatamzadeh, Firouzi, & Jaymand, 2019; Tajdaran, Chan, Gordon, & Borschel, 2019). PLGA is among the most commonly used

biodegradable polymers for tissue engineering and drug delivery and is approved by the FDA for human clinical applications (Chun, Yoo, Yoon, & Park, 2004; Mundargi, Babu, Rangaswamy, Patel, & Aminabhavi, 2008). Fibrin is a fibrous biopolymer, produced from cross-linked fibrinogen and thrombin with factor XIIIa. Besides its necessary role in blood coagulation and hemostasis, it has been proven to be beneficial in tissue engineering and wound healing and used as a tissue sealant and delivery tool in nerve repair (Amrani, Diorio, & Delmotte, 2001; Asmani et al., 2013; Sameem, Wood, & Bain, 2011).

Tacrolimus, contained in PLGA microspheres and suspended in fibrin gel, is released by sustained diffusion through surface erosion of the PLGA microspheres and then through the gel. In previous studies, the optimal neuroregenerative doses of tacrolimus *in vitro* have been reported to be 0.01 ng/mL and 100 ng/mL and these were therefore investigated in this study (Tajdaran, Chan, Zhang, et al., 2019). Previous studies have shown that locally delivered tacrolimus enhances neurite extension within a wide dose range (Tajdaran, Chan, Zhang, et al., 2019). Microspheres containing 0.01 ng/mL of tacrolimus showed depletion by 13 days and were not found to be feasible for further *in vivo* experiments. The delivery system containing 0.01 ng/mL tacrolimus is thought to have a faster depletion rate due to saturation of the PLGA-tacrolimus microspheres within a small volume of fibrin gel. The relatively lower volume of gel reduces the barrier to diffusion and thus, increases the degradation and drug release rates. Previous research has corroborated that the release rate of tacrolimus from the microspheres decreased with an increase in the PLGA content (Kojima et al., 2015). PLGA empty microspheres also resulted in a faster depletion of the fibrin gel. As the empty microspheres degrade, they create localized cavities within the fibrin gel. This

increases the porosity of the gel over time, thus leading to a faster bulk gel degradation rate (Gu, Sun, Papadimitrakopoulos, & Burgess, 2016).

Microspheres containing 100 ng/mL demonstrated two phases of release. An initial steady release up to day 15 was followed by a more rapid release rate beyond day 15. The release profile observed is in line with a previous study by Tajdaran and colleagues (Tajdaran et al., 2015). In the first 15 days, tacrolimus was being released from the outer superficial layers of the PLGA microspheres. As depletion occurs, the core of the PLGA microspheres starts releasing tacrolimus. PLGA (50:50 lactide:glycolide) microspheres, as used in this study, demonstrated loss of 50% molecular weight by 15 days (Shive & Anderson, 1997). This implicates that the total degradation would occur in approximately 30 days and explains the second phase of release. The use of 100 ng/mL of tacrolimus microspheres is feasible for *in vivo* applications and could be recognized as a novel delivery tool when combined with MSCs. Moreover, its ease in application may overcome limitations that are associated with current delivery methods of MSCs, such as intra-neural microinjection (Fairbairn, Meppelink, Ng-Glazier, Randolph, & Winograd, 2015; Mathot et al., 2019; Wang, Lu, Peng, Hu, & Wang, 2017).

The degradation process of PLGA through hydrolysis of ester bonds generates acidic byproducts. However, these products have not shown toxicity *in vitro* nor *in vivo* (Kang & Singh, 2001). Tacrolimus released from the fibrin gel construct exerts substantial bioactivity during the entire period of release (Tajdaran et al., 2015). Pharmacokinetic studies show the half-life of tacrolimus to be approximately 12 hours, therefore 94-97% of the drug will have been eliminated around day 15-16 in the 0.01 ng/mL tacrolimus fibrin gel constructs as tacrolimus showed depletion by 13

days (Wallemacq & Verbeeck, 2001). Some tacrolimus may be present at day 15, however, the fibrin gel construct most likely has been degraded completely by day 28 as the mean time to resorption is 10-14 days for Tisseel® fibrin gel (Tisseel). While day 28 may not represent the effect of tacrolimus on stem cell viability directly for 0.01 ng/mL (Figure 3), it does shed light on the effect of the generated acidic byproducts that are released in the PBS supernatant. Since no significant differences are found in cell viability, we confirm that neither the PLGA microspheres including tacrolimus nor the acidic byproducts that are generated during degradation are toxic for stem cell viability.

MSCs have great translational potential in regenerative medicine given their availability and potential for multilineage differentiation, and they have been demonstrated to improve nerve regeneration by the secretion of local neurotrophic factors e.g. nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), pleiotrophin (PTN), growth associated protein 43 (GAP43) and peripheral protein 22 (PMP22) (Cooney et al., 2016; Mathot, 2021; Parekkadan & Milwid, 2010; Rbia et al., 2020; Rbia et al., 2019). Their therapeutic effect, however, relies on the persistent viability of the transplanted cells, which may be impaired by host immune rejection (Li et al., 2018). Very few studies have investigated the cytotoxicity of drug delivery systems to transplanted stem cells (Li et al., 2018; Nih et al., 2017). Li and colleagues have found that free tacrolimus in hydrogel impaired the viability of stem cells in a higher concentration, while tacrolimus loaded in nanoparticles did not (Li et al., 2018). This finding is hypothesized to be due to the sustained release of tacrolimus over time, without any initial drug burst release (Tajdaran, Chan, Shoichet, et al., 2019). Overall, a hydrogel scaffold that combines local tacrolimus and MSC delivery could overcome the systemic side effects of immunosuppressants, while inducing a local

immunotolerant environment, extending the survivability of stem cells and providing neuroregenerative effects.

In a rat sciatic nerve defect model, local delivery of encapsulated tacrolimus in fibrin hydrogel has been proven to enhance nerve regeneration with little to no drug detection in other vital organs (Tajdaran, Chan, Shoichet, et al., 2019). Moreover, local tacrolimus has demonstrated to significantly improve the regeneration of retrogradely labeled motor and sensory neurons in unprocessed fresh nerve allografts compared to rats in which a 10-mm nerve gap was reconstructed with nerve allografts without tacrolimus (Kevin J. Zuo et al., 2020). Future *in vivo* rat studies are needed to evaluate the combined effect of locally delivered tacrolimus and MSCs on histologic and functional nerve regeneration (Figure 6). This delivery method could be easily translated to clinical practice for the enhancement of direct nerve repair or segmental nerve gap reconstruction using nerve grafts.

Conclusions

The local environment of the nerve following injury is critical to successful regeneration and could be modulated to enhance peripheral nerve regeneration. In this study, a fibrin gel-based drug delivery system for sustained and localized tacrolimus release combined with MSCs was used to investigate cell viability and feasibility. Encapsulation of 100 ng/mL tacrolimus in microspheres combined with MSCs in hydrogel did not demonstrate cytotoxic effects on the transplanted stem cells. A hydrogel scaffold that combines both local tacrolimus and MSCs delivery could overcome the systemic side effects of immunosuppressants, while inducing a local immunotolerant

environment, extending the survivability of stem cells, and providing neuroregenerative effects in peripheral nerve injury.

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Tables

Experiment A	Description	Time points (days)	Outcome measurements

I	Tacrolimus in hydrogel incubation	1-35 days	• Tacrolimus release profiles
			  ○ Cumulative release (%)
			  ○ Cumulative mass released (µg)
			  ○ Mass release (µg)

II	MSC incubation in collected PBS containing tacrolimus	At day 7, 15, and 28	• MSC viability
			  ○ Live/dead staining
			  ○ Metabolic activity (MTS assay)

Table 1. Experimental design for Experiment A.
MSCs: Mesenchymal stem cells. PBS: Phosphate buffered saline. MTS assay: (3-(4,5-dimethylthiazol-2-yl) −5-(3-carboxymethoxyphenyl) −2-(4-sulfophenyl) −2H-tetrazolium) assay.

Experiment B	Description	Concentration tacrolimus	Outcome measurements

I	MSC + hydrogel	-	• MSC Presence
			  ○ CD90 staining
			• MSC viability
			  ○ Live/dead staining
			  ○ Metabolic activity (MTS assay)

II	MSC + PLGA empty microspheres	-	
III	HIST 0.01	0.01 ng/mL	
IV	HIST 100	100 ng/mL	

Table 2. Experimental design for Experiment B.
MSCs: Mesenchymal stem cells. PLGA: Poly(lactic-co-glycolic acid). HIST: hydrogel infused with stem cells and tacrolimus. MTS assay: (3-(4,5-dimethylthiazol-2-yl) −5-(3-carboxymethoxyphenyl) −2-(4-sulfophenyl) −2H-tetrazolium) assay.

Figure legends

Figure 1. In vitro 100 ng/mL tacrolimus (FK506) release profiles from fibrin gel. *Cumulative mass release of 100 ng/mL tacrolimus in percentage (A) and mass (in μg, B) are depicted over a course of 35 days. The mass release profiles of tacrolimus from poly(lactic-co-glycolic acid) (PLGA) microspheres depicts the sustained drug release up to 35 days, with a step-wise increase at 15 days and steady release rate thereafter.*

Figure 2. Microscopic images of mesenchymal stem cells (MSC) cultured in tacrolimus. *Live/Dead staining of MSCs in stem cell media (control) combined with collected phosphate buffered saline (PBS) from releasing tacrolimus microspheres at 7, 15 and 28 days. MSCs were incubated in PBS collected from 0.01 ng/mL and 100 ng/mL tacrolimus containing poly(lactic-co-glycolic acid) (PLGA) microspheres. Scale bar is set at 100 μm.*

Figure 3. Viability of mesenchymal stem cells (MSC) cultured in PBS containing tacrolimus. *MSC viability was assessed using Live/Dead staining (A) and MTS assay (B) to compare MSC viability when cultured in collected phosphate buffered saline (PBS) from 0.01 ng/mL and 100 ng/mL tacrolimus containing poly(lactic-co-glycolic acid) (PLGA) microspheres at 7, 15 and 28 days. Cell viability was expressed as a ratio of the viability of MSCs cultured in stem cell media without tacrolimus and given as the mean ± SEM.*
SEM = Standard error of the mean.

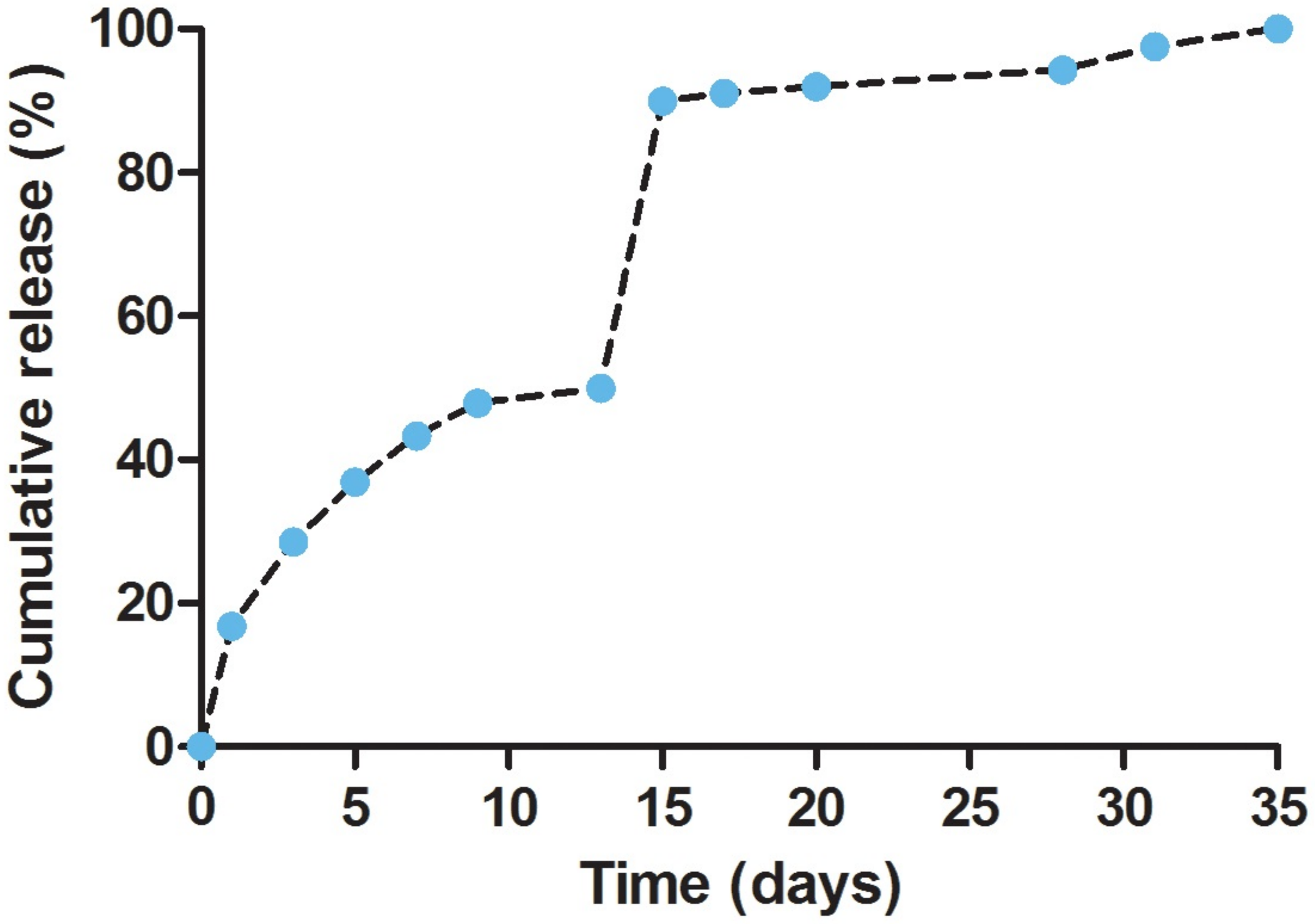
Figure 4. Microscopic images of mesenchymal stem cells (MSC) cultured in hydrogel groups at 72 hours. *MSCs are cultured in stem cell media (control) combined with (i) hydrogel, (ii) poly(lactic-co-glycolic acid) (PLGA) empty microspheres, (iii) hydrogel infused with stem cells and tacrolimus (HIST) in 0.01 ng/mL and (iv) 100 ng/mL. MSC characterization is confirmed using staining against CD90 (in green), a canonical MSC surface marker and combined with DAPI to denote cell nuclei (in blue). The lower row depicts the microscopic images obtained after Live/Dead staining of MSCs. Stem cells in hydrogels are distributed differently compared to control, hence the difference in density and scale bars. Scale bars are set at 100μm and denote different magnification.*

Figure 5. Viability of mesenchymal stem cells (MSC) and tacrolimus combined in hydrogel. *MSC viability was assessed using Live/Dead staining (A) and MTS assay (B) to compare MSC viability when combined with (i) hydrogel, (ii) poly(lactic-co-glycolic acid) (PLGA) empty microspheres, (iii) hydrogel infused with stem cells and tacrolimus (HIST) in 0.01 ng/mL and (iv) 100 ng/mL. *denotes depletion of gel in empty PLGA microspheres (no tacrolimus), which prevented gel analysis for cell viability. Cell viability was expressed as a ratio of the viability of MSCs cultured in stem cell media without tacrolimus and given as the mean ± SEM.*
SEM = Standard error of the mean.

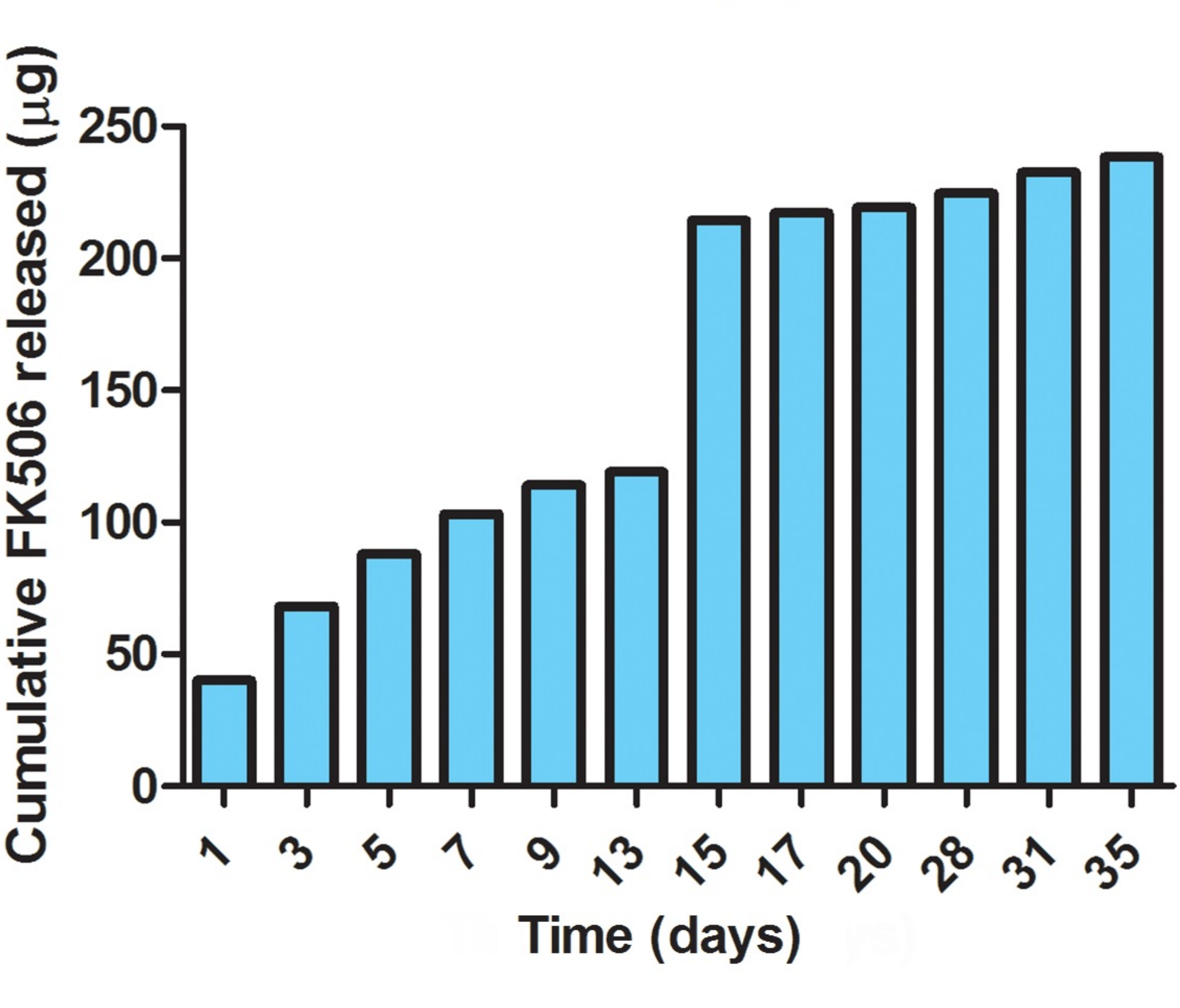
Figure 6. Schematic overview of the hydrogel scaffold that combines local tacrolimus and MSCs delivery. *This delivery tool could overcome the systemic side effects of immunosuppressants, while inducing a local immunotolerant environment, extending the survivability of stem cells and providing neuroregenerative effects. This construct does not harm cytotoxicity of transplanted stem cells and is feasible for future in vivo experiments. (Used with permission of Mayo Foundation for Medical Education and Research. All rights reserved).*

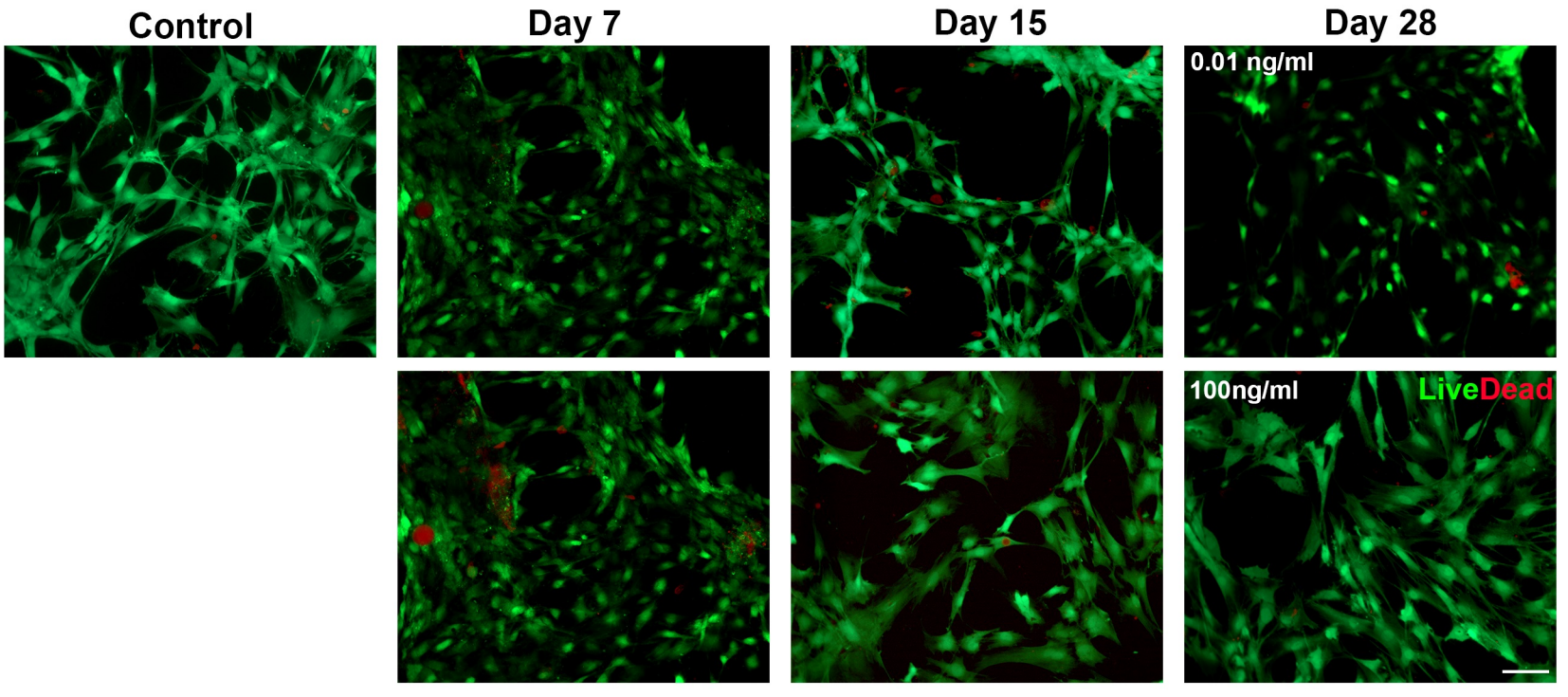
Supplementary Online Material Figure 1. In vitro 0.01 ng/mL tacrolimus (FK506) release profiles from fibrin gel. *Cumulative mass release of 0.01 ng/mL tacrolimus in percentage (A) and mass (in ng, B) are depicted. The mass release profiles of tacrolimus from poly(lactic-co-glycolic acid) (PLGA) microspheres depicts a drug release up to 13 days.*

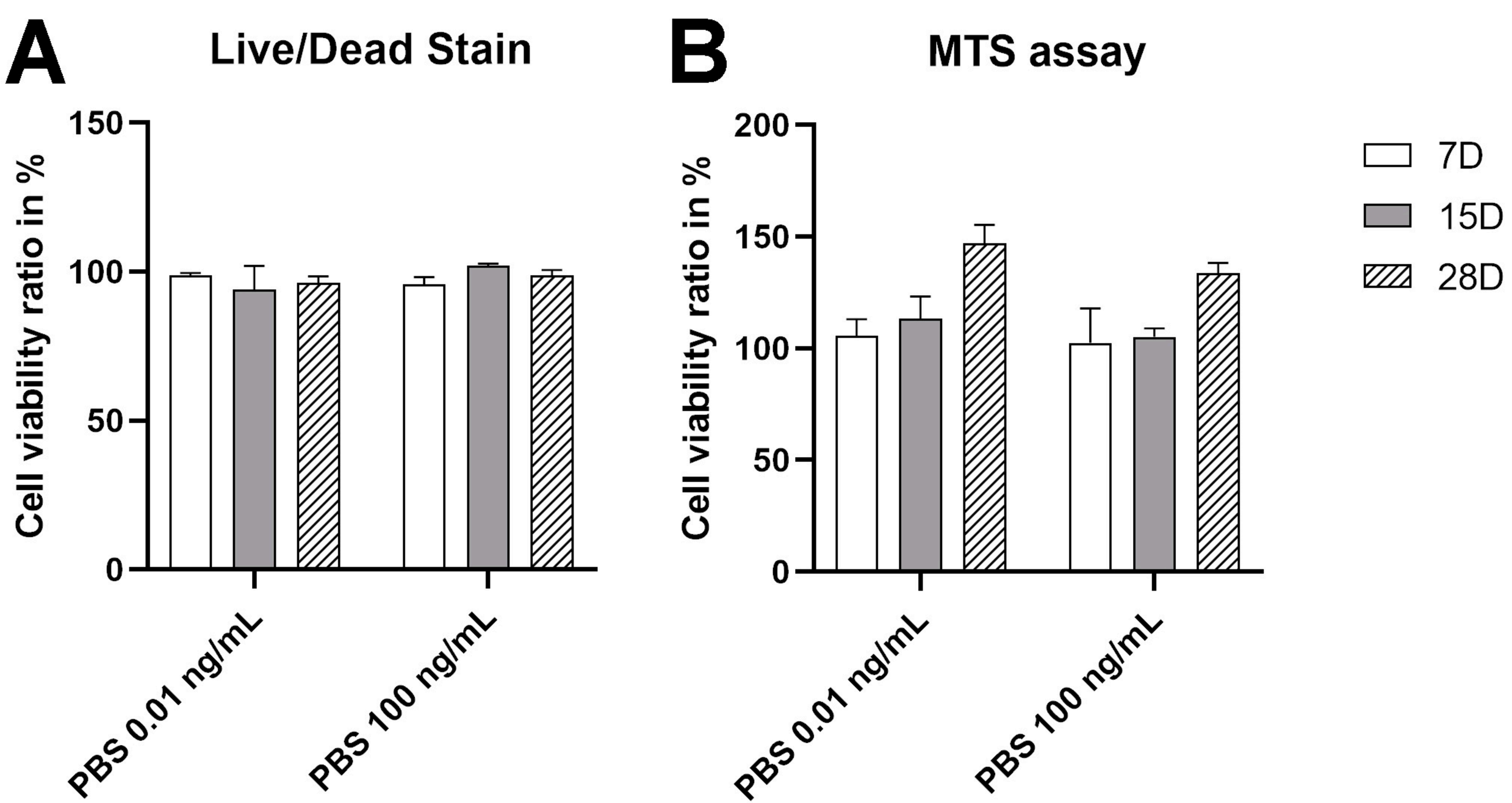
A

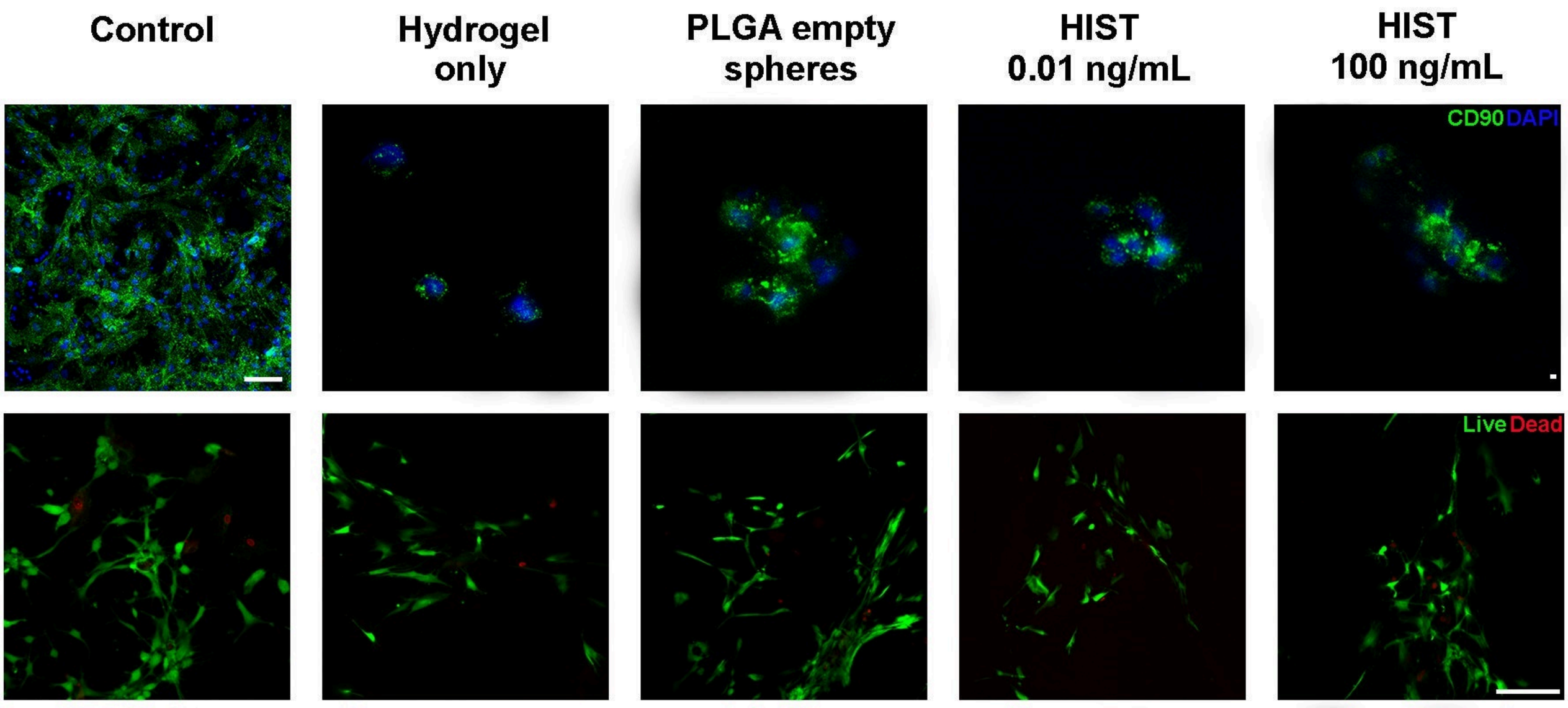

Cumulative release (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Time (days)

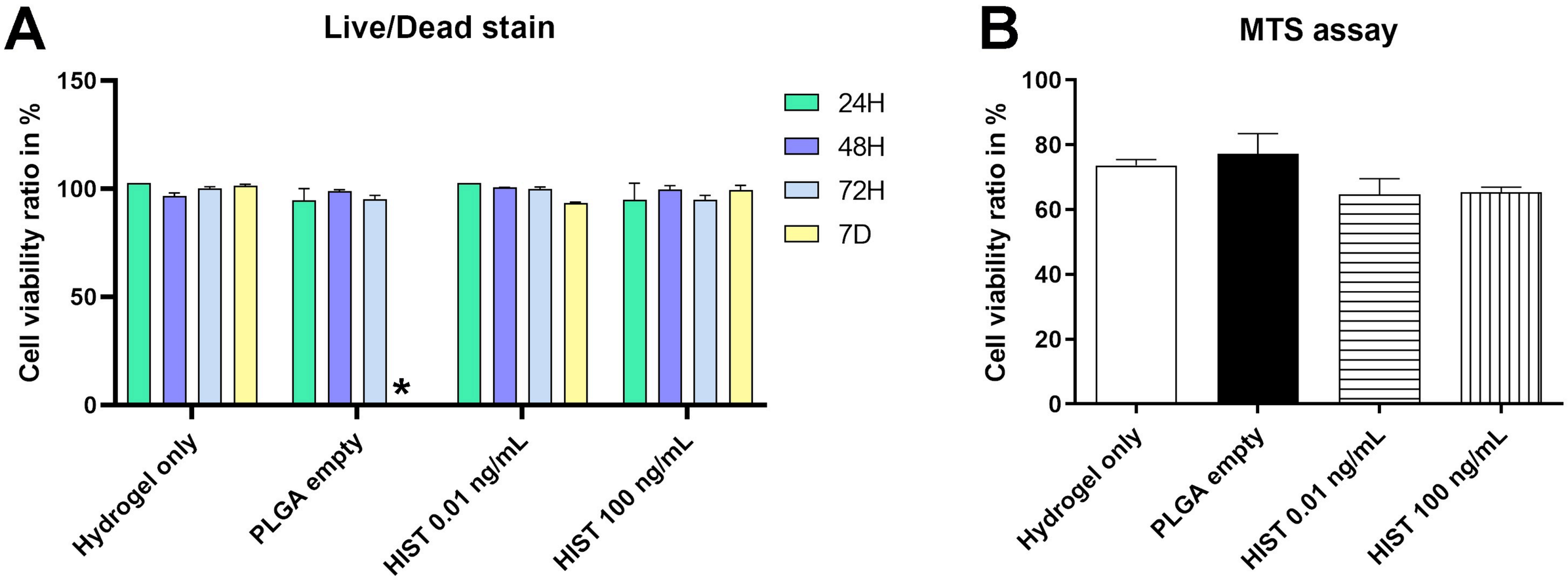
B

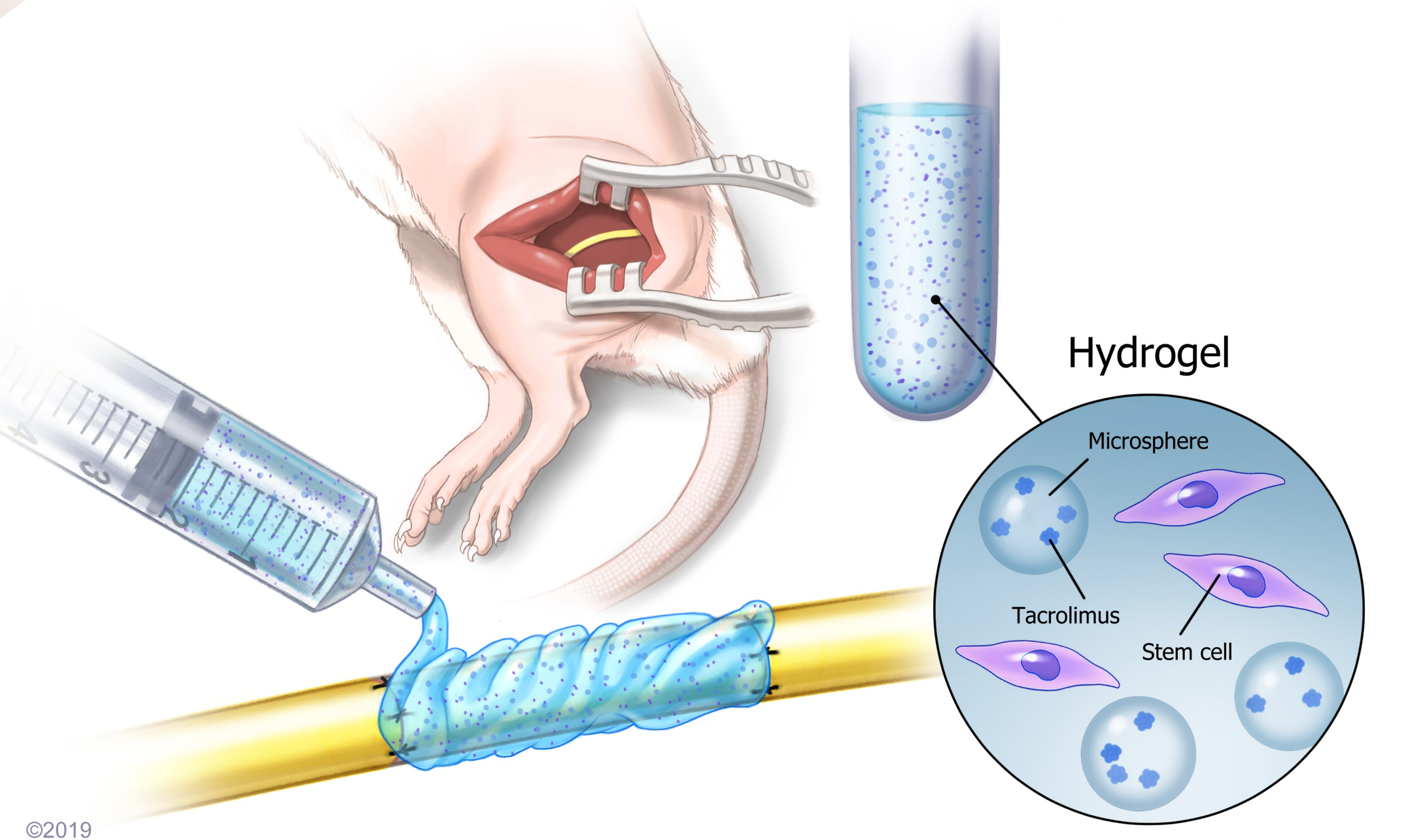

Cumulative FK506 released (μg)
250
200
150
100
50
0
1
3
5
7
9
13
15
17
20
28
31
35
Time (days)


Control
Day 7
Day 15
Day 28
0.01 ng/ml
100ng/ml
LiveDead

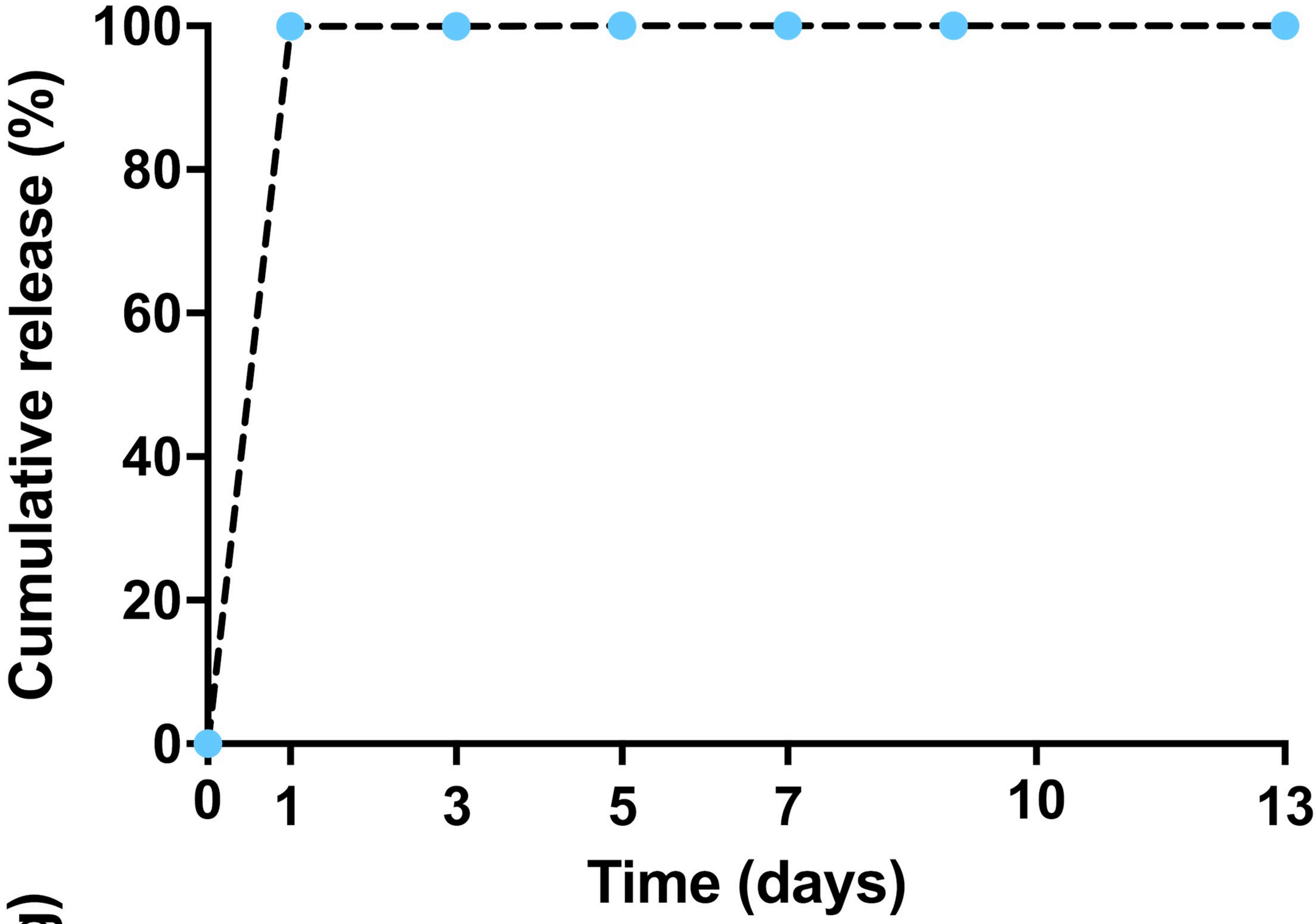

A
Live/Dead Stain
Cell viability ratio in %
150
100
50
0
PBS 0.01 ng/mL
PBS 100 ng/mL
B
MTS assay
Cell viability ratio in %
200
150
100
50
0
PBS 0.01 ng/mL
PBS 100 ng/mL
7D
15D
28D


Control
Hydrogel only
PLGA empty spheres
HIST 0.01 ng/mL
HIST 100 ng/mL
CD90 DAPI
Live Dead


A
Live/Dead stain
Cell viability ratio in %
150
100
50
0
*
24H
48H
72H
7D
Hydrogel only
PLGA empty
HIST 0.01 ng/mL
HIST 100 ng/mL
B
MTS assay
Cell viability ratio in %
100
80
60
40
20
0
Hydrogel only
PLGA empty
HIST 0.01 ng/mL
HIST 100 ng/mL



A


Cumulative release (%)
100
80
60
40
20
0
0
1
3
5
7
10
13
Time (days)

B

Cumulative FK506 released (ng)
88.0
87.5
87.0
86.5
86.0
80
40
0
1
3
5
7
9
13
Time points (days)