

Controlled release of GAG-binding Enhanced Transduction (GET) peptides for sustained and highly efficient intracellular delivery

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Abstract

Controlled release systems for therapeutic molecules are vital to allow the sustained local delivery of their activities which direct cell behaviour and enable novel regenerative strategies. Direct programming of cells using exogenously delivered transcription factors can by-pass growth factor signalling but there is still a requirement to deliver such activity spatio-temporally. We previously developed a technology termed GAG-binding enhanced transduction (GET) to efficiently deliver a variety of cargoes intracellularly, using GAG-binding domains which promote cell targeting, and cell penetrating peptides (CPPs) which allow cell entry. Herein we demonstrate that GET system can be used in controlled release systems to mediate sustained intracellular transduction over one week. We assessed the stability and activity of GET peptides in poly(DL-lactic acid-co-glycolic acid) (PLGA) microparticles (MPs) prepared using S/O/W double emulsion method. Efficient encapsulation (~65%) and tailored protein release profiles could be achieved, however intracellular transduction was significantly inhibited post-release. To retain GET peptide activity we optimised a strategy of co-encapsulation of L-Histidine, which may form a complex with the PLGA degradation products under acidic conditions. Simulations of the polymer microclimate showed that hydrolytic acidic PLGA degradation products directly inhibited GET peptide transduction activity, and use of L-Histidine significantly enhanced released protein delivery. The ability to control the intracellular transduction of functional proteins into cells will facilitate new localised delivery methods and allow approaches to direct cellular behaviour for many regenerative medicine applications.

Keywords: Intracellular transduction; Controlled release; GAG-binding enhanced transduction (GET), CPP, PLGA, L-Histidine.

1. Introduction

Successful regenerative medicine strategies often rely on exquisite control of the biological microenvironment and supplementation of extrinsic therapeutic molecules to direct cellular behaviour. Cellular processes like migration, proliferation, adhesion and differentiation occur in response to chemical cues present within the microenvironment such as interaction with extracellular matrix (ECM) and stimulation with morphogens or growth factors [1, 2]. These macromolecules trigger intracellular signal transduction pathways, with complex cross-talk between the cascades and eventual programming of cell behaviour through transcription factors [2-4]. Our recent work has shown that recombinant transcription factors can be expressed, purified and delivered efficiently to control cell behaviour for regenerative medicine strategies [5]. Such systems will allow the biological control of cells and promote regeneration of tissues if it is supplied at a functional dose, duration and location to be effective.

Various methodologies have been developed to deliver therapeutic proteins intracellularly [6-8] with the aim to direct differentiation of somatic [9, 10] or stem cells [11]; effectively bypassing the need for growth-factor stimulation. Cell penetrating peptides (CPPs) can be tethered to the protein of interest [12-14] and these trigger endocytosis-mediated uptake when they interact with the cell membrane [6-8]. Even though CPPs significantly increase cellular uptake, their activity requires them to be in vast extracellular excess to drive endocytosis, with the quantities required to elicit changes in cell behaviour at micromolar scale. To overcome this inefficiency, we recently described a new technology named glycosaminoglycan (GAG) enhanced transduction (GET) [5]. GET technology is a series of novel fusion proteins that couple a membrane-docking peptide to heparan

sulfate glycosaminoglycans (GAGs) with a PTD. We demonstrated enhanced intracellular transduction upon coupling CPPs with a GAG-binding peptide (the composite peptide termed GET peptides). Functional quantities of many cargos including fluorescent proteins, transcription factors and enzymes were successfully delivered using GET peptides with significant increase in functional delivery compared to conventional CPP-mediated delivery and using sub-micromolar doses. Furthermore we could create 3D gradients of these proteins which mediate cell programming dose-dependently [15].

In vivo, therapeutic proteins can be delivered by subcutaneous and intramuscular administration, or intravenous infusion. However, most proteins possess short biological half-lives in bodily fluids which means that frequent injection regimens at high dosages are required for sustained effect. Moreover, systemic delivery by nature does not allow targeted or localised delivery of proteins, meaning off-target effects and significant amounts of the administered therapy is miss-targeted. In order to overcome these limitations, biodegradable polymers have been used to control the release of therapeutic proteins locally at the disease site. These controlled release approaches can be powerful as they extend the duration of activity, overcoming the need for frequent administration, and also implantation at the therapeutic site allows localized delivery to the tissue of interest [16-19].

Many studies have employed biodegradable polymer microparticles (MPs), such as poly-ε-caprolactone, polylactide (PLA), polyglycolide (PGA) and their co-polymer poly-lactic and -glycolic acid (PLGA), to mediate controlled release [20-24]. These MPs protect the therapeutic cargo from degradation and themselves progressively degrade to release the cargo spatio-temporally. PLGA has been extensively studied to encapsulate proteins and has been used in long-term delivery systems, with

injectable MPs formulations being used to deliver growth factor to promote osteogenic differentiation for bone repair. Importantly these proteins have been successfully delivered over a period of one month using PLGA MPs [25, 26]. However, very few proteins can be easily delivered with this release approach [27], as they can undergo physical or chemical degradation during MPs manufacturing of these formulations [28-34]. Accordingly, delivery formulations have to be designed to protect protein activity during encapsulation and release. Furthermore, MPs loading needs to be efficient as delivered formulations have to provide functional quantities of protein at the site of injection.

During MPs fabrication, many factors can affect protein stability, such as protein instability at the water – organic interface using double emulsion methods such as water-in-oil-in-water (W/O/W) [30], protein–polymer interaction [35], and local acidity created within degrading polymer matrix [36-38].

Several approaches have been developed to protect protein activity during MPs fabrication, such as adding excipients to the inner aqueous phase or removing the need for the aqueous phase entirely [29, 39-41]. Excipients (such as Cyclodextrin and PEG [19, 29, 40, 41]) can be added to compete with the therapeutic protein for exposure at the water-organic interface. A study by Morita et al [42] suspended dry protein particles in the organic solvent with the aid of PEG, which entirely removes the water–organic interface, and is termed solid-in-oil-in-water (S/O/W) double emulsion.

In order to overcome local acidity limitation, many groups studied pH neutralization in the PLGA microenvironment [19, 43-45]. Various low soluble basic salts were studied, such as magnesium hydroxide [45] and Poly-L-Histidine coupled with PEG [19]. The use of these excipients resulted in neutralized acidic microenvironment

within the PLGA MPs system. Other issues can arise if the therapeutic proteins interacts with the polymer used [19]. Protein–polymer interactions can occur through hydrogen bonding, and through hydrophobic or electrostatic interactions [19]. Furthermore, proteins may interact with the degradation products of the polymer; PLGA degrades through an acid-catalysed hydrolysis and produces carboxylic acid molecules which can interact with cationic molecules. Electrostatic and hydrophobic interactions are also common factors for protein–polymer complexation [46-48].

In this study, we demonstrate efficient encapsulation, and release of a GET-tagged reporter protein from PLGA MPs. Using a S/O/W double emulsion method we were able to fabricate MPs and eliminate the water–organic interface which significantly effects protein stability and also removes the need for harsh homogenisation during manufacture. PEG was used to pre-form protein nanoparticles, which enabled organic solvent resuspension and reduced non-specific protein polymer interaction. By using a fluorescently labelled GET-peptide (red fluorescent protein; mR), we were able to directly assess the stability and activity of the GET peptides post-release. We observed that PLGA degradation resulted in accumulation of acidic products in the microenvironment, which can interact with the positively charged GET peptides. Therefore we employed L-Histidine as a proton-scavenger to enhance protein stability during release. L-Histidine is a positively charged amino acid at pH ≤ 6 , and our data suggests it can compete with the GET protein to complex with carboxylic acid PLGA degradation products and help retain GET-mediated transduction activity. Our optimized controlled release system maintains GET-protein transduction activity post-release and allows sustained release of transduction-active GET protein for 7 days. This approach will allow the localized and sustained delivery of GET-tagged

therapeutic molecules and create the potential to target intracellular transduction by MPs-mediated controlled release.

2. Materials and methods

2.1. GET protein and fabrication of MPs

CPP (eight arginine, 8R) and heparin binding domain; HBD (P21), which compose the Glycosaminoglycan – binding enhanced transduction (GET) system were tagged with red fluorescent protein (mRFP1) producing P21mR8R protein. GET-mR was expressed and purified from *Escherichia coli* using GST affinity purification as previously described [5].

Poly (D,L-lactide-co-glycolide, (PLGA) LA:GA ratio: 50:50; Mw 52kDa, Evonik Industries, USA) MPs were formed using solid-in-oil-in-water (S/O/W) emulsion as previously described by Morita et al. [42], with some modifications. Briefly, 1mg of P21mR8R, was mixed with 0.5mg PEG 6000 (Sigma-Aldrich, UK) and freeze dried overnight. For MPs containing L-Histidine, 0.25mg L-Histidine (Sigma-Aldrich, UK) was mixed with the P21mR8R–PEG solution and then freeze dried. Blank MPs were prepared by freeze drying 0.5mg PEG 6000 only. 15% (w/v) PLGA in dichloromethane (DCM, Fisher, UK) (200mg of PLGA was dissolved in 1.5 ml DCM) was added to the freeze dried powder, until completely dissolved. The solution was then mixed by vortexing. For MPs prepared with phosphate buffered saline (PBS), 200µL PBS (Gibco, UK) was added to the solution and vortexed to produce homogenous emulsion. 4ml of 0.25% (w/v) methylcellulose (Sigma-Aldrich, UK) solution was then added to the mixture and vortexed again. The resultant emulsion was then poured into 400 ml of distilled water; and the resultant (S/O/W) emulsion was left stirring for 3 hours to allow DCM evaporation. The hardened MPs were then

collected by centrifugation and washed three times by distilled water (DW). The obtained MPs were then freeze dried for 24 hours.

2.2 MP characterization: Size, charge and morphology

A thin layer of MPs were placed onto an adhesive stub and gold-coated using a SCD030 gold sputter coater (Balzers Union Ltd., Lichtenstein), for five minutes at 30 mA. The morphology of the MPs was assessed by scanning electron microscopy (SEM), using a JEOL 6060L imaging system (JEOL Ltd., Hertfordshire, UK) with the accelerating voltage set to 10 kV.

The mean MPs diameter and size distribution were measured using a Coulter LS230 particle size analyser (Beckman, UK). MPs were suspended in HPLC grade water, under stirring conditions. Size distribution data was collected after achieving an obscuration value of 8 – 12%.

The zeta potentials of the MPs was measured using NanoZS instrument (Malvern, UK) in PBS buffer of various pH values (3, 5 & 7).

2.3 MPs characterization: Determination of protein loading (loading efficiency)

Measurement of protein content in the MPs was conducted as previously described [49, 50] (modified from a technique proposed by Sah [51] and Morita et al. [52]). 10 mg of PLGA MPs were added to 750 μ L DMSO and shaken at room temperature for one hour; 2150 μ L of 0.02% (w/v) SDS in 0.2 M NaOH was then added and incubated for one hour at room temperature. The Micro BCA protein assay kit was used to measure the total protein content and compared against a freshly generated standard curve of P21mR8R. 150 μ L sample volume was mixed with 150 μ L BCA working reagent and incubated at 37°C for 2 hours. Absorbance at 562nm was then measured using a plate reader (Infinite M200, Tecan UK Ltd., Reading, UK).

2.4 MPs characterization: GET protein release

30 mg MPs were resuspended in 1 ml Growth media (GM) or HBSS. Samples were rocked on a 3D shaker (Gyrotwister, Fisher Scientific UK Ltd) at 5 rpm in a humidified incubator at 37°C. At defined time points, samples were centrifuged and the supernatant was removed from the MPs and replaced with fresh GM or HBSS. The supernatants were assayed for GET and total protein content using fluorometric analysis for mRFP1 (excitation: 584 nm; emission: 620 nm; GM samples) and μ BCA assay kit (HBSS samples). Released protein contents were compared against freshly generated standard curve of P21mR8R.

2.5 Cell Culture

NIH3T3 mouse fibroblast cells were maintained in Growth Media (GM; DMEM with 10% (v/v) foetal calf serum (FCS; Sigma) media supplemented with 2mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin). Cells were maintained in a humidified tissue-culture incubator at 37°C and with 5% CO₂ atmosphere.

2.6 GET peptides stability assessment

GET peptide stability in different pH conditions was evaluated. The pH of cell culture media was adjusted (pH 3, 5 & 7) using one of the following solutions; 1M glycolic acid, 1M acetic acid, 1M phosphoric acid, 1M hydrochloric acid and 1M sulfuric acid. 200 μ g/ml P21mR8R (with or without L-Histidine; 200 μ g/ml) solution was prepared and incubated at 37°C. At defined time-points, samples were taken and diluted 1:10 in fresh cell culture media to neutralise the sample to pH 7.2. Protein was then transduced into cells by addition to pre-plated NIH3t3 cells.

2.7 GET peptides transduction activity

Protein transduction activity was evaluated using pre-plated NIH 3t3 cells, as previously described [5]. Briefly, 1×10^5 cells/well (in 24-well plates) were plated and attached for 4 hours, culture media was then replaced with the media containing P21mR8R protein (protein released from MPs (section 2.4) or protein samples from stability test (section 2.6)). After 18 hours, cells were washed with PBS, trypsinized and fixed in 4% (w/v) PFA for flow cytometry analysis.

2.8 GET peptides extraction from PLGA MPs using acetone precipitation

Protein transduction activity was evaluated after the manufacturing process of PLGA MPs. This was done to test the effect of PLGA MPs manufacturing process on protein activity. Known quantity of MPs were vortexed with cold acetone and incubated for 1 hour at $-20\text{ }^{\circ}\text{C}$. The solution was then centrifuged at $12,000\text{g}$ for 10 minutes. The acetone was then removed, and the pellet was allowed to dry from the remaining acetone. Growth media was then added to the protein pellet. The protein activity was evaluated by adding the protein to plated NIH 3t3 cells, as mentioned in section 2.7.

2.9 GET peptides interaction with blank PLGA MPs

Known quantities of P21mR8R were mixed with blank PLGA MPs, for 30 minutes at 37°C in 1ml PBS. The solution was centrifuged ($10,000\text{ g}$ for 10 mins) and the free P21mR8R quantity in the supernatant was measured using fluorometric analysis for mRFP1 (excitation: 584 nm; emission: 620 nm).

2.10 GET peptides interaction with PLGA degradation product

GET peptides interaction with PLGA degradation products was tested by suspending blank PLGA MPs in media for one week at 37°C . The solution was centrifuged

(10,000 g for 10mins) to collect PLGA degradation products in the supernatant. The supernatant was mixed with P21mR8R and different quantities of L-Histidine (P21mR8R : L-Histidine ratios were 1:0, 1:0.1, 1:0.25, 1:0.5 & 1:1). Protein was then transduced into cells by addition to pre-plated NIH3t3 cells as detailed in section 2.7.

2.11 Flow Cytometry and Microscopy

Fixed cells were analysed on a Beckman Coulter Astrios EQ Flow Cytometer using green laser (561 nm). (20,000 cells; gated on live cells by forward/side scatter). Median fluorescence was used for statistical analyses with background from untransduced cells subtracted and values were then calculated in ratio to the experimental control. For microscopy, cells were imaged with fluorescence microscope using a TRITC filter (Leica DM IRB).

2.12 Gel Electrophoresis

For determination of protein molecular weights after incubation in media of various pH values, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Novex 10% Tris-Glycine mini protein gel (Invitrogen) was applied following manufacturer's instructions. Gels were stained with Coomassie blue (Invitrogen) and de-stained before imaging.

2.13 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism (version 6). The statistical significance was determined using one way ANOVA with Sidak post hoc test. Results were considered significant at $p < 0.05$, mean values are given plus or minus the standard deviation (SD).

3. Results

3.1 Fabrication of porous high-encapsulation PLGA MPs

We fabricated PLGA MPs using an optimised S/O/W double emulsion process and encapsulated a GET-tagged fluorescent reporter (P21mR8R) [5] (Figure 1A). The process was optimised without employment of homogenizer, as our results showed loss of protein transduction activity upon homogenisation (i.e. homogenised protein at 9000rpm for 2 minutes showed a relative transduction activity of $13\% \pm 5.2\%$ compared to non-homogenised protein).

MPs prepared using the S/O/W methodology showed spherical structure with smooth, non-porous surface (Figure 1B). The mean diameter of these MPs was $45.2 \pm 9.7 \mu\text{m}$, with average loading efficiency of $73.2 \pm 7.6 \%$ (Figure 1 C&D). We hypothesized that porous MPs will result in accelerated protein release from MPs. It was known that addition of PBS during MPs fabrication would produce porous MPs [53]. As predicted PBS addition resulted in porous structure of the MPs (Figure 1B). The mean diameter for these MPs found to be $41.5 \pm 10.5\mu\text{m}$, with average loading efficiency of 65.7% (Figure 1 C&D). PBS addition therefore did not significantly affect MPs size or encapsulation efficiency. Therefore we created GET-encapsulated MPs (with high encapsulation efficiency) which can be engineered to be porous by simple addition of PBS during fabrication.

3.2 Porous MPs allow sustained release of GET proteins

We next assessed the cumulative protein release from P21mR8R-containing MPs (Figure 2 A&B) for the previously mentioned formulations (Figure 1). Protein release was quantified by two methods, biochemically using μBCA assay (spectrophotometry) and intensity of the released mRFP1 fluorescence (fluorometry). Non-porous PLGA MPs showed an initial burst release (around $20\% \pm 4.2$ of total

loaded) within the first day, with no further detectable protein was released even with a further 21 days of incubation (μ BCA detection limit is 0.5 μ g/ml) (Figure 2 A&B) (the release is expected to continue after day 21 as previously shown [50]). It was expected that the porous formulations would have more efficient protein release. The release profile obtained from MPs with PBS-created pores (PLGA-PBS) showed a similar burst release (on day 1, 18% \pm 3.7) but this level was retained producing a continuous protein release over 5-7 day period. More than 90% \pm 7.9, cumulative release, could be achieved after 10 days (Figure 2 A&B), after which little further protein was released. We were therefore able to demonstrate a controlled release of P21mR8R from porous-MP (PLGA-PBS) formulations.

3.3 GET transduction activity is inhibited during sustained release formulations

We showed efficient release of GET proteins over a 7 day period using S/O/W fabricated PLGA porous-MPs (PLGA-PBS). Next we aimed to demonstrate the released GET protein retained its full transduction activity by comparing released P21mR8R to non-encapsulated controls. Released GET-protein (from porous PLGA-PBS MPs) was collected over the 7 day time course and was incubated with NIH3t3 cells to assess internalisation and transduction ability. Importantly we compared transduction to non-encapsulated controls diluted to the same final concentration. Incubated cells were then evaluated for transduction by fluorescence microscopy and flow cytometry analyses (Figure 2 C-E).

Fluorescent microscopy showed significant GET protein internalisation for samples released from PLGA-PBS MPs in the first day (Figure 2C). Flow cytometry results showed that the protein released at the first day has a relative transduction activity

above $85\% \pm 12.3$ (Figure 2 D&E). However, GET-protein released from these MPs (PLGA-PBS) showed gradual loss of protein transduction activity with relative transduction activity below $35\% \pm 4.7$ at day 7 (Figure 2E). Overall, activity of the fully released GET-protein over the 7 days is therefore significantly inhibited (with only $\sim 51\% \pm 9.5$ transduction is realised verses the non-encapsulated P21mR8R), and less than $35\% \pm 4.7$ activity towards the end of the release profile. We hypothesized it may be possible that the released P21mR8R protein was interacting with PLGA or its degradation products, inhibiting the interaction with cell membranes and endocytosis. This inhibitory phenomenon would restrict the use of GET-protein in this controlled release system and we believed further optimisation was required to improve the relative transduction activity from released protein.

3.4 Co-encapsulation of L-Histidine during fabrication of porous PLGA MPs

It is known that acidic microenvironment results from PLGA degradation can affect protein stability [19, 54]. It was suggested that P21mR8R can be affected by either the acidic microenvironment during controlled release from PLGA or its transduction activity was being inhibited by interaction with PLGA or its degradation products. We hypothesized that addition of a proton-scavenger (such as L-Histidine) may improve the activity of the released protein from MPs. Our hypothesis was that the carboxyl groups liberated from PLGA degradation may interact with the positively charged GET peptides, inhibiting transduction. L-Histidine, a positively charged amino acid at $\text{pH} < 6$, is expected to neutralize the acidic pH and act as a scavenger and bind to the carboxyl groups. We therefore generated porous PLGA MPs containing L-Histidine (0.25% w/w). L-Histidine MPs prepared with addition of PBS, have the average diameter and loading efficiency of $42.3 \pm 9.1\mu\text{m}$ and $67.4 \pm 6.3\%$ respectively. Using SEM and particle sizing, we were able to determine that incorporation of L-Histidine

did not affect the morphology, size and the loading efficiency of MPs, as shown in the representative SEM images (Figure 1B) and size distribution and loading efficiency results (Figure 1 C&D), respectively. This demonstration was important as we showed it is possible to add excipients (such as L-Histidine) directly to the formulation without affecting MPs physical properties.

3.5 L-Histidine has no effect on sustained release of GET proteins from porous MPs

To confirm that the same release kinetics are retained with incorporation of L-Histidine we repeated the analyses of P21mR8R release over time. Incorporation of L-Histidine to the formulation (PLGA-PBS-Histidine) did not change the release profile significantly (Figure 2 A&B), MPs showed gradual and continuous release similar to plain porous-MPs (PLGA-PBS), with around $83\% \pm 9.8$ cumulative release, after 10 days. We were therefore able to demonstrate a controlled release of P21mR8R from porous-MP (PLGA-PBS) formulations with or without the addition of a potential excipient (L-Histidine; PLGA-PBS-Histidine).

3.6 L-Histidine protects GET transduction activity in sustained release formulations

We next assessed whether the addition of L-Histidine to MPs can improve the transduction activity of the released GET-protein (PLGA-PBS-L-Histidine formulations). Flow cytometry results showed that the protein released at the first day has a relative transduction activity above $85\% \pm 8.6$, similar to that of PLGA-PBS MPs (Figure 2 D&E). However, GET-protein released from PLGA-PBS-Histidine MPs retained its relative transduction activity above 80% on all studied time points (Figure 2E). This observation clearly demonstrates that L-Histidine addition to porous-PLGA MPs significantly prevents the loss of GET transduction activity during protein

release. The mechanism by which L-Histidine helps retain GET transduction activity could be through negating internal acidic pH within the degrading MPs, or there could be direct interaction between PLGA or its degradation products and P21mR8R which would be inhibited in presence of L-Histidine.

3.7 Understanding the mechanism of L-Histidine activity to promote GET transduction in controlled release formulations.

3.7.1 GET activity is not influenced by MPs fabrication

We initially aimed to ascertain if the fabrication process itself or the release environment was the inhibitory factor for GET transduction activity post-release. Initially we formulated MPs and extracted the GET protein directly after fabrication using acetone. Acetone extraction did not affect GET protein activity and was an effective way to rapidly recover encapsulated P21mR8R from MPs. The results showed that the relative transduction activity of the protein extracted from MPs is around $88.3 \pm 9.3\%$, compared to fresh protein. This clearly demonstrated that the manufacturing process is not affecting the GET peptides activity but the release process was inhibiting protein transduction.

3.7.2 GET activity is inhibited by PLGA degradation products at neutral pH

We identified the controlled release as the inhibitory process on GET transduction activity. We next assessed any possible interaction between the GET peptides and the PLGA degradation products and its impact on GET peptides transduction activity. When PLGA degrades by hydrolysis it releases carboxyl group containing molecules which could interfere with GET transduction activity. We studied the GET peptides activity in presence of PLGA degradation products collected from PLGA degradation in media. The pH of the media upon GET peptides addition was around 6.0 ± 0.2 .

Fluorescence microscopy showed that there was very low transduction activity for P21mR8R added to cells with degraded PLGA supernatants (Figure S1 A), flow cytometry showing a relative transduction activity of around $34\% \pm 8.9$ (Figure S1 B). This data therefore confirms that during controlled release PLGA degradation products will have an inhibitory effect of GET protein transduction activity.

3.7.3 L-Histidine prevents GET peptide interaction with PLGA degradation products

The previous results showed that PLGA degradation product is inhibiting GET peptides activity (Section 3.7.2), in order to assess L-Histidine effect on GET-PLGA degradation product interaction, the experiment was repeated with various quantities of L-Histidine being incorporated with P21mR8R. Addition of L-Histidine resulted in enhanced transduction activity, as the L-Histidine is expected to interact with the carboxylic group-containing PLGA degradants. Samples of 1:0.1 ratio (P21mR8R:L-Histidine) showed enhanced transduction activity, compared to samples with no L-Histidine (Figure S1 A), however, only around $55\% \pm 4.3$ transduction activity could be achieved (Figure S1 B). For samples prepared with addition of L-Histidine $> 25\%$ of the P21mR8R quantity, fluorescent microscopy and flow cytometry results showed comparable protein transduction activity at all time points (Figure S1 A), with relative transduction activity above 80% could be achieved (Figure S1 B). This clearly demonstrates the role of L-Histidine in maintaining the activity of GET peptides in presence of PLGA degradation products.

3.7.4 GET peptides interact directly with PLGA MPs at neutral pH

PLGA MPs are negatively charged [55]. Blank PLGA MPs charge was measured at various pH values (3, 5 & 7). The results showed that the PLGA MPs are negatively charged at all studied pH values (table S1). As GET peptides are positively charged

[5] (the calculated isoelectric point (pI) for P21 and 8R is 10.9 and 12.9 respectively (table S2) [56]) it is expected that P21mR8R interacts with the negatively charged PLGA MPs. In order to evaluate the interaction between the GET peptides and PLGA MPs, we assessed the direct interaction between P21mR8R and PLGA MPs in a pulldown assay. P21mR8R was mixed with various quantities of blank PLGA MPs. After incubation, MPs were removed by centrifugation and the loss of soluble P21mR8R (binding to PLGA) was assessed by fluorometry. This assay was designed to determine if there was any direct interaction of P21mR8R with intact PLGA polymer. The results demonstrated that at neutral pH less P21mR8R protein remains free in the supernatant with increasing MPs quantities (i.e. only 20% of the protein quantity could be detected when mixed with 25mg of PLGA MPs) indicating that the P21mR8R interacts with PLGA polymer in the MPs (Figure S2 A). Many interaction types can be associated in P21mR8R binding to PLGA MPs, but the main type to control the interaction, in presence of positively charged peptides, is electrostatic interaction [57]. Hydrophobic interaction might be affecting the adsorption of P21mR8R on the surface of PLGA MPs due to the fact that the PLGA is hydrophobic. However this interaction is considered negligible [46, 48, 57].

3.7.5 Free Carboxyl groups inhibit GET/PLGA interaction.

We have shown that P21mR8R is interacting with PLGA MPs at neutral pH, however, as PLGA degrades it releases lactic and glycolic acid molecules which decrease the pH. We hypothesized that rather than pH disrupting the P21mR8R/PLGA interaction that the presence of carboxyl groups may affect PLGA/P21mR8R interaction. We repeated our interaction assay at lower pH value (~3).

Interestingly, at pH 3, all the protein remained in the supernatant, and there was no significant interaction with the PLGA MPs (Figure S2 A). This was unexpected as we hypothesized that in a low pH microenvironment (which would be similar to that generated in hydrolysing PLGA during the release process) that P21mR8R would still interact with PLGA polymers, as our results suggested that the PLGA will be negatively charged at this pH (table S1). This observation was important, we demonstrated that P21mR8R can directly interact with PLGA, but that this can be disrupted by the presence of carboxylic acid.

It was imperative that we counteract the negative influence of carboxylic acid binding of P21mR8R if PLGA polymer was to be used for successful controlled release of GET peptides. We therefore tested excipient addition to this assay aiming to disrupt P21mR8R interaction with carboxylic groups. Applying the same conditions in presence of L-Histidine resulted in GET peptides interaction with MPs at neutral pH (Figure S2 B), with only around 20% of the protein quantity could be detected, which is expected as the L-Histidine is not positively charged at this pH. Interestingly, at pH 3, the positively charged L-Histidine appeared to act as a scavenger for the carboxyl groups from the added carboxyl groups and P21mR8R retained its ability to interact with the negatively charged PLGA MPs. We therefore conclude that P21mR8R can interact with both PLGA directly (most likely electrostatically) and also with carboxylic groups (which can disrupt the PLGA interaction). By using L-Histidine we could retain P21mR8R/PLGA interaction, most likely through a sequestering of free carboxylic acid groups by positively charged L-Histidine (Figure S2 B).

3.8 GET peptides stability in various pH conditions simulating PLGA MP microenvironment

3.8.1 GET peptides activity is inhibited in presence of carboxyl acid group

In order to fully understand the mechanism by which GET-protein transduction activity is inhibited post-release, we aimed to model the conditions experienced by the P21mR8R protein inside the MPs without the fabrication. During protein release the degrading PLGA is hydrolysed, releasing glycolic and lactic acid (carboxyl group containing molecules) into the microenvironment and it is progressively acidified. To test the effect of such acid groups and the overall pH have on retention of GET-transduction activity, P21mR8R was incubated in cell culture media of various pH values (3, 5 & 7), pH adjusted using either 1M lactic acid, 1M glycolic acid or 1M acetic acid solutions. After incubation, samples were neutralized by dilution and protein transduction activity was evaluated, using NIH 3t3 cells (fluorescence microscopy and flow cytometry). The neutralization process is important as all proteins were transduced into cells at the same neutral pH (pH 7.2), therefore this directly assesses the effect of pH during the incubation, with transduction as a readout of functionality. Protein storage, sampling and transduction into cells were done as diagrammed in Figure S3 A. Fluorescence microscopy showed significant protein internalization, for samples stored at pH 3, at day 1 compared to controls, however, protein transduction was undetectable for day 7 samples (Figure 3 A, Figure S4 A) this can be seen in samples of all studied solutions; lactic acid, glycolic acid and acetic acid. Flow cytometry results confirmed a loss in transduction for day 7 samples (Figure 3 C & D, Figure S4 E), and gradual loss of relative transduction activity over the time-points, reaching values below 30% at day 7 (Figure 3 C & D, Figure S4 E). Interestingly, fluorescent microscopy showed protein transduction for

samples stored at pH 5 & 7 at all studied time points (Figure 3 C & D, Figure S4 E). For pH 5, flow cytometry results confirmed this with relative transduction activity above 80% of non-treated controls at all studied time points. For pH 7, flow cytometry results showed minimal decrease in relative transduction activity with values around 60% at all studied time points (Figure 3 C & D, Figure S4 E). Importantly both pH 5 and 7 data shows high levels of activity is retained, even with extended culture (7 days). This is unlike activity seen at pH3 samples which is progressively decreased until very low levels are left at day 7 (~25%). Therefore high concentration carboxylic acid inhibits the activity of GET transduction over time. Importantly this is not due to stability of the protein as fluorescence of the P21mR8R was not affected by these conditions.

3.8.2 L-Histidine prevents carboxylic acid-mediated inhibition of GET transduction

To determine if L-Histidine could promote GET-protein transduction in high carboxylic acid/low pH as for MPs-released protein, we repeated the pH incubation experiments in the presence of L-Histidine. As before, protein was stored in cell culture media of various pH values (3, 5 & 7); adjusted using either 1M lactic acid, 1M glycolic acid or 1M acetic acid. Unlike P21mR8R alone, L-Histidine (200µg/ml) containing samples (either for fluorescence microscopy and flow cytometry analyses) did not lose P21mR8R transduction activity for all the studied pH conditions including those at pH 3 (Figure 3 B – D, Figure S4 F). Protein relative transduction activity was above 60% for all the studied conditions (Figure 3 C & D, Figure S4 F) and therefore it was clear that the presence of L-Histidine during incubation prevented the loss of GET transduction activity.

3.9 L-Histidine dose is important to preserve GET transduction

Our previous analyses employed one concentration (200µg/ml) of L-Histidine to negate the negative effect of carboxylic acid on GET peptides transduction. Next we assessed the dose requirement for this activity. P21mR8R (200µg/ml) with different quantities of L-Histidine (0 – 200µg/ml) was incubated at pH3 in acetic acid-adjusted cell culture media. Fluorescence microscopy and flow cytometry demonstrated gradual loss of protein transduction activity for samples with no L-Histidine (Figure 4A & B). Samples containing 20µg/ml L-Histidine showed enhanced transduction activity, compared to samples with no L-Histidine (Figure 4A & B), however, only 50% transduction activity could be achieved after 3 days (Figure 4C). For samples prepared with addition of > 50µg/ml of L-Histidine, fluorescent microscopy and flow cytometry results showed comparable protein transduction activity at all time points (Figure 4 A&B), with relative transduction activity above 60% could be achieved in all time points (Figure 4C). Therefore 50µg/ml L-Histidine was sufficient to mediate the maximum effect to negate the inhibitory effect of carboxylic acid. This represents 25% of the P21mR8R quantity.

4. Discussion

Optimized porous PLGA MPs for efficient GET-protein controlled release. GET reporter protein, P21mR8R, release from PLGA MPs prepared using S/O/W was found to be very low, with ~20% released as a burst in the first day, and no further release up to 21 days (released protein quantities below detection limit were eliminated). We accelerated the protein release by incorporating PBS during the formulation. PBS worked as a porogen, which increased MPs porosity and generated channels within and on the surface of the MPs [53]. These pores

increased the release to above ~90% in 7 days period. Importantly, PBS addition did not change the protein encapsulation efficiency nor the MP size, which provides a robust method to deliver GET proteins over a 7 day period. It is worth noting that the release study was performed for up to 21 days. A previous study within our group [50] showed a similar formulation began another lower phase of release after 21 days until day 35. Our aim was to develop a delivery system to release the majority of contents over 7 days (~90% release) using PBS as a porogen, however it is likely that lower protein levels are still being released but this is either below the sensitivity of detection or after the 21 day sampling period.

GET peptides stability in PLGA. It was clear that P21mR8R was stable upon extraction and initial release from PLGA MPs. Therefore we expected the released protein would retain transduction activity over the release period. To investigate this, P21mR8R was encapsulated in PLGA MPs, the released quantity was monitored and its activity to transduce into cells assessed. The results were compared to experimental control (equivalent fresh protein quantity). The results showed loss of protein transduction activity with time (i.e. the protein released at the first day showed 80% activity, while the protein released at day 7 was below 30%) (Figure 2). The protein could be detected using fluorometric analysis, which suggests that the fluorescent part of the protein (mR) was intact but the GET peptides, enhancing the protein internalization in the cells, were no longer functional. This may be attributed to protein hydrolysis (i.e. detachment of the GET peptides (P21 & 8R) from the fluorescent part (mR)), or interaction between the negatively charged PLGA (or its degradation product) with the positively charged GET peptides, which would prevent the interaction between GET peptides and cell membrane heparan sulfate.

GET peptides interaction with PLGA and its degradation products. The possible interaction between GET peptides and PLGA MPs was assessed, the results showed that the positively charged P21mR8R is interacting with the negatively charged PLGA MPs. Moreover, the P21mR8R showed loss of transduction activity when exposed to PLGA MPs degradation products.

Simulating GET peptides instability in the polymer. During protein release, acidic microenvironment usually develops with PLGA MPs as a result of poly ester hydrolysis, this results in carboxylic acid accumulation [19]. To test the effect of this acidic environment on protein transduction activity, we simulated GET peptides instability in PLGA microclimate. P21mR8R was exposed to different conditions of pH (3, 5 & 7) for one week. Of the studied conditions, only highly acidic conditions (pH 3) showed loss of protein transduction activity. Gel electrophoresis results showed that P21mR8R degraded at these conditions (figure S11). The results showed gradual loss of P21mR8R transduction activity (Figure 3), which was experienced during protein release from PLGA MPs. On the other hand, samples stored at $\text{pH} \geq 5$, protein relative transduction activity was above 60% at all studied time points (Figure 3). The common factor between the simulating study and the PLGA MPs is the carboxylic acid accumulation in the media. The same protein behaviour was observed in both studies, which confirms that GET peptides are affected by carboxylic acid.

GET peptides stability in presence of other acids. GET peptides stability in acidic conditions was studied using different acids (Weak acids: lactic acid, glycolic acid, acetic acid & phosphoric acid. Strong acids: hydrochloric acid (HCl) & sulfuric acid). The results showed that GET peptides gradually lose the activity in presence of weak acids, but immediately in presence of strong acids.

Developing a hypothesis for mechanism of instability of encapsulated GET peptides. Low pH usually develops in PLGA MPs as a result of accumulation of acidic degradants. From our simulation studies, a highly acidic environment (~pH 3) was needed to inactivate the protein transduction activity. The results suggested that the negatively charged carboxylic acid groups, which results from PLGA degradation, will interact with the positively charged GET peptides, preventing them from binding to the cell membrane and consequently preventing protein internalization. A schematic showing the proposed interaction/mechanism between GET peptides and the carboxyl group liberated during PLGA degradation is shown in Figure 5 (A & C).

Developing a stabilization approach using L-Histidine. Since GET peptides activity is compromised in presence of carboxylic acid group, the intended approach is to scavenge this group by adding a molecule that will compete with the protein in interaction with carboxylic acid. GET peptides are positively charged, and it is expected that they will interact with the negatively charged molecules. L-Histidine is positively charged amino acid at pH ≤ 6 . L-Histidine is expected to compete with the P21mR8R in interacting with the carboxylic group. Consequently, to test whether the L-Histidine could enhance GET peptides activity at low pH, we have repeated the pH simulation study, in presence of L-Histidine (Figure 3). L-Histidine was added in quantities that will not affect the solution pH (Figure S8). As expected, P21mR8R showed enhanced transduction activity, in presence of L-Histidine, compared to samples without L-Histidine. Moreover, P21mR8R maintained its structure in presence of L-Histidine at low pH (figure S11). Various pH conditions were studied (5 & 7), with and without L-Histidine, and the same protein behaviour was noticed,

which suggests that the L-Histidine works at low pH only, due to its charge, and the presence of L-Histidine along with the protein, at higher pH, will not alter its activity.

L-Histidine works in presence of weak acids but not strong acids. Results showed that L-Histidine can negate the effect of weak acids on GET peptides but not strong acids. This may be due to the fact that strong acids are denaturing/deaminating GET peptides.

L-Histidine quantity is critical in maintaining GET peptides activity. In order to study the effect of the L-Histidine quantity on the protein transduction activity, different quantities of L-Histidine were examined (0 – 200 µg/ml). The maximum protein activity could be achieved at ratios higher than 1:0.25 w/w (protein: L-Histidine).

PLGA MPs containing L-Histidine as a stabiliser for GET peptides. In order to validate our findings on actual PLGA MPs, L-Histidine was encapsulated with P21mR8R inside PLGA MPs. Protein release was monitored, with protein release behaviour comparable, with and without L-Histidine, exhibiting a burst release of around 20%, then continuous and complete release up to 7 days. The released protein transduction activity was significantly higher for MPs prepared with addition of L-Histidine, compared to MPs without L-Histidine. Protein relative transduction activity was above 80% at day 7, with addition of L-Histidine, compared to 30% without L-Histidine. A schematic showing L-Histidine role in maintain GET peptides transduction activity is shown in Figure 5 (B & D).

5. Conclusion

A robust method of fabricating protein-loaded MPs was optimized, using S/O/W emulsion technique. L-Histidine was used as a stabiliser to maintain the GET system

activity during release from MPs. Incorporation of L-Histidine in PLGA MPs did not change the protein release profile. The presence of L-Histidine in the MPs effectively enhanced the protein stability in acidic conditions caused by degradation of PLGA. The maintenance of the protein stability by incorporating L-Histidine in PLGA MPs is strong evidence supporting the hypothesis that the GET peptides are interacting with acidic degradation products of the PLGA and the addition of L-Histidine is a successful strategy.

The advantage of this delivery system is to provide localized release of GET-tagged therapeutic molecules with minimised risk of systemic dosing that may lead to non-targeted activity. This delivery technology has potential to be valuable in the controlled delivery of various potent therapeutic molecules, coupled with GET peptides, which can be used for drug delivery and regenerative medicine applications.

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7. Author Contributions. H-A.M.A-A. and J.E.D conceived and initiated the project. H-A.M.A-A. and J.E.D designed and performed experiments. L.T. provided reagents and technical expertise. J.E.D supervised the project. H-A.M.A-A and J.E.D wrote the paper.

8. Competing Financial Interests. The authors declare no competing financial interests.

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10. Figure Legends

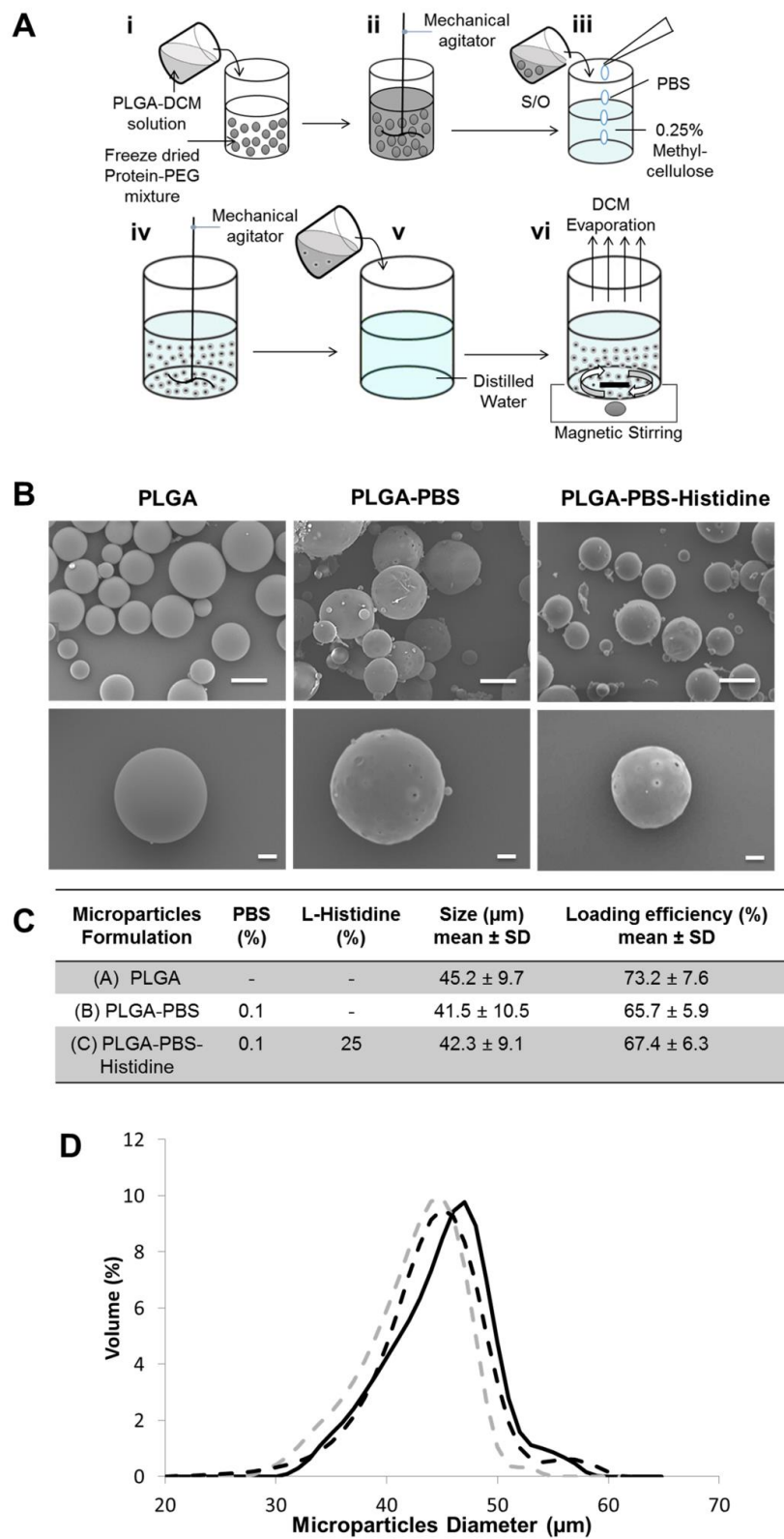


Figure 1. MP manufacturing process and characterization; SEM and size analyses. MPs were fabricated (15% (w/v) PLGA 50:50), loaded with P21mR8R (0.5% (w/w)), mixed with different quantities of PBS and L-Histidine (1) No PBS, no L-Histidine. (2) 0.1% (w/w) PBS, no L-Histidine. (3) 0.1 % (w/w) PBS, 25% (w/w) L-Histidine. (A) Schematic showing the solid-in-oil-in-water double emulsion method of PLGA MPs manufacturing (B) SEM analyses for MPs (scale bars represent 50 μ m on top images, and 10 μ m on bottom images). (C) Summary of size characterization and corresponding loading efficiencies (n = 3). (D) Representative size distribution of the different PLGA MPs formulation, (black line) No PBS, no L-Histidine, (dashed grey line) 0.1% (w/w) PBS, no L-Histidine, (dashed black line) 0.1 % (w/w) PBS, 25% (w/w) L-Histidine.

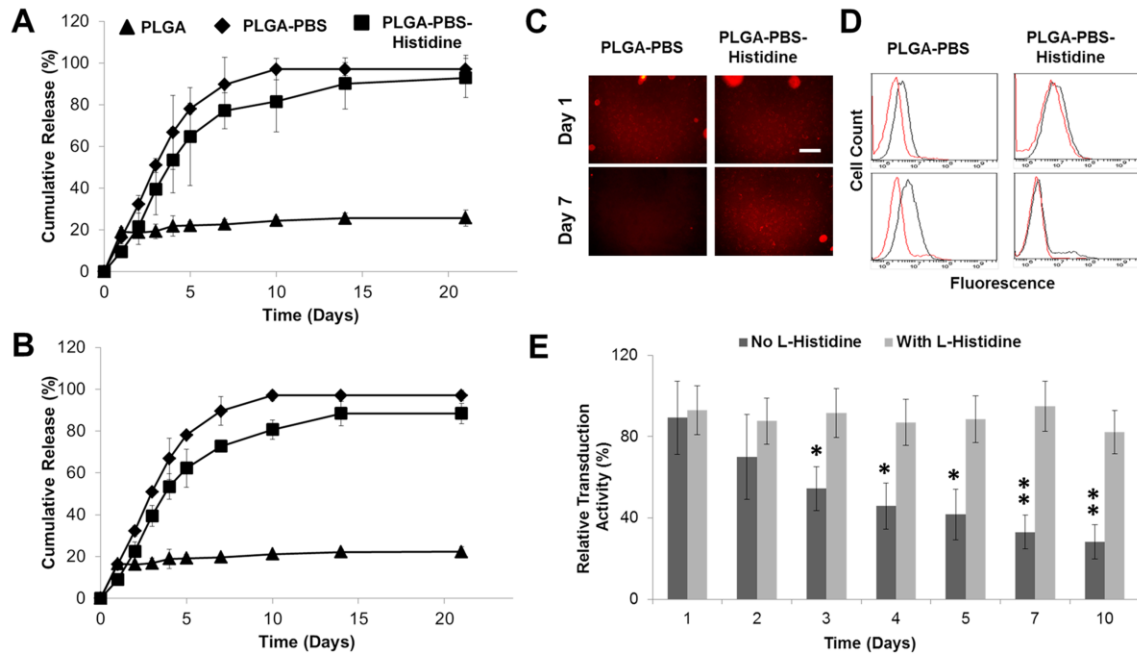


Figure 2. *In vitro* release profiles of P21mR8R from PLGA MPs (pH 7.4 at 37 °C) and transduction activity of the released P21mR8R. MPs were prepared using S/O/W method (15% (w/v) PLGA), loaded with P21mR8R (0.5% (w/w)), mixed with different quantities of PBS and L-Histidine (see Figure 1 for MPs characteristics). (A) Protein was quantified using fluorescence intensity (B) protein was quantified using Micro BCA assay kit. (▲) No PBS, no L-Histidine. (◆) 0.1% (w/w) PBS, no L-Histidine (■) 0.1 % (w/w) PBS, 25% (w/w) L-Histidine. Error bars indicate SD (n = 3). Transduction activity of the released protein was assessed by addition to plated NIH3t3 cells. This study was performed for two different formulations PLGA-PBS and PLGA-PBS-Histidine. (C) Fluorescence microscopy images of the NIH3t3 cells treated with the sampled P21mR8R (Scale bar 10µm). (D) Flow cytometry histograms showing cellular uptake of P21mR8R after application of the samples to NIH3t3 cells. The red histograms are the P21mR8R samples, while the black histograms are the experimental control. (E) Relative biological activity of the

sampled P21mR8R. Protein transduction activity was compared to the experiment control. Error bars indicate SD (n = 3). * is $p < 0.05$, ** is $p < 0.01$.

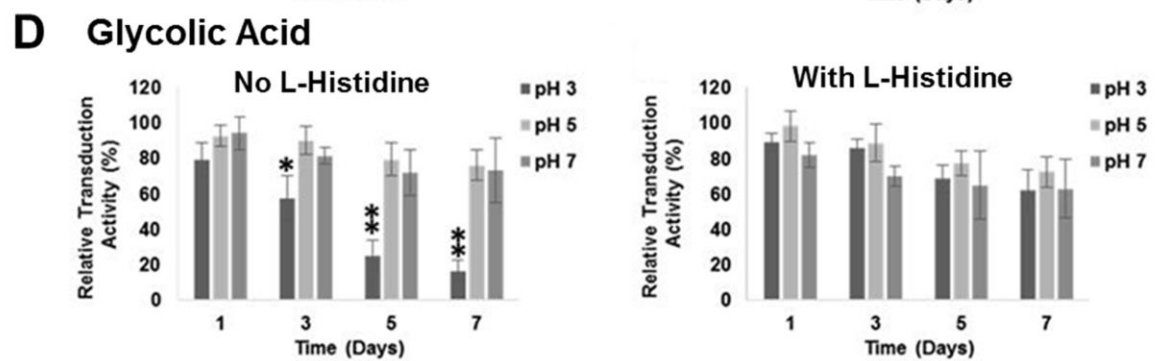
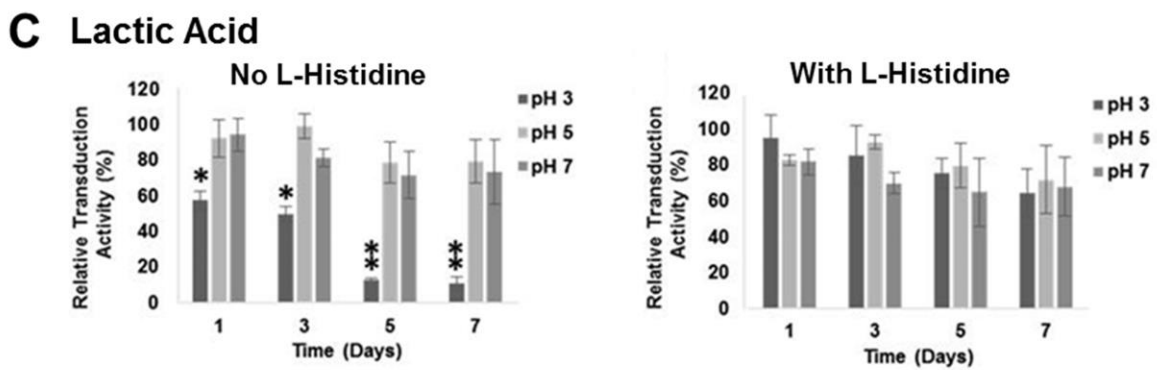
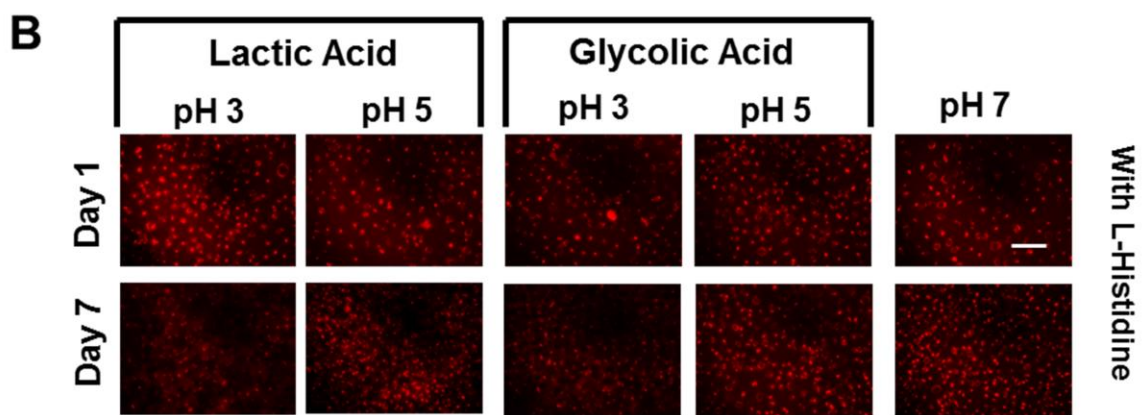
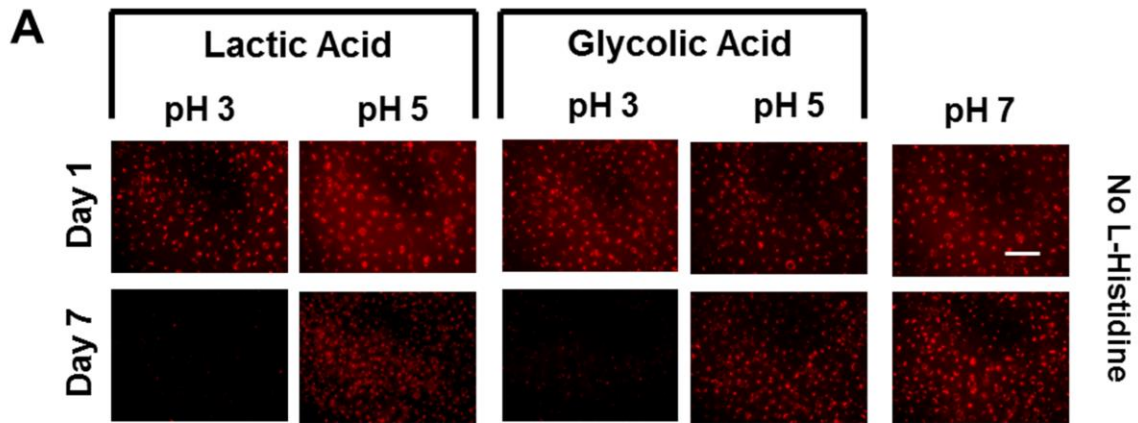


Figure 3. GET-peptide stability at various pH values mimicking the PLGA microenvironment. P21 mR8R stability in cell culture media of various pH values (3, 5 & 7), with and without L-Histidine. pH was adjusted using either 1M lactic acid solution or 1M glycolic acid solution. Protein sample was transduced into cells by addition to plated NIH3t3 cells. Fluorescence microscopy images of the NIH3t3 cells treated with the protein samples. (A) P21mR8R only and pH adjusted using 1M lactic acid or 1M glycolic acid solutions (Scale bar 10 μ m). (B) P21mR8R and L-Histidine and pH adjusted using 1M lactic acid or 1M glycolic acid solution (Scale bar 10 μ m). Relative transduction activity of the samples (C) P21mR8R with and without L-Histidine and pH adjusted using 1M lactic acid solution (D) P21mR8R with and without L-Histidine and pH adjusted using 1M glycolic acid solution. Protein transduction activity was compared to the experiment control. Error bars indicate SD (n = 3). * is p<0.05, ** is p<0.01.

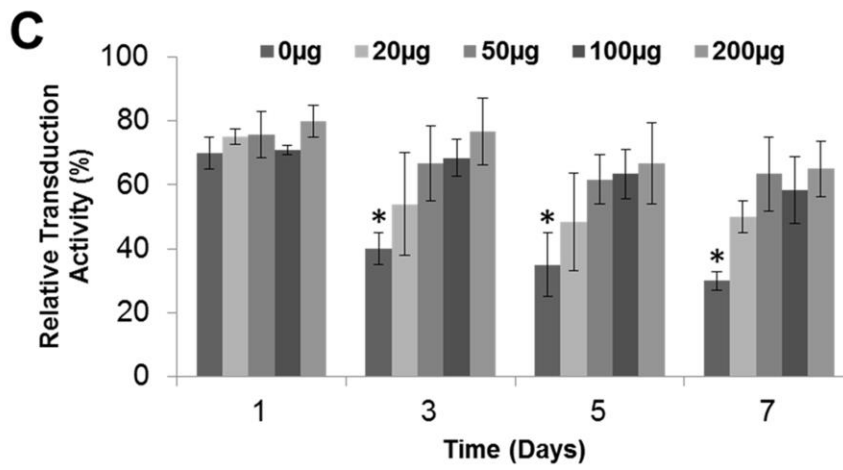
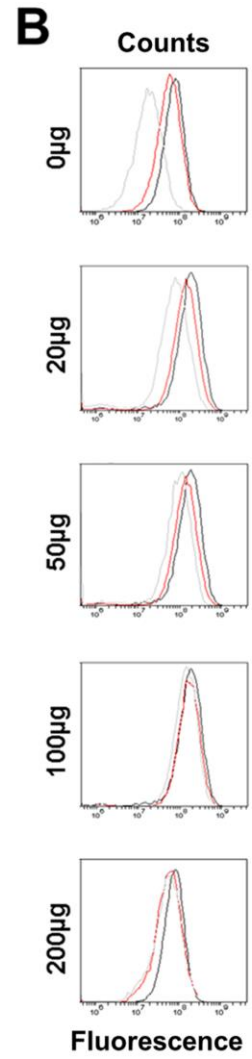
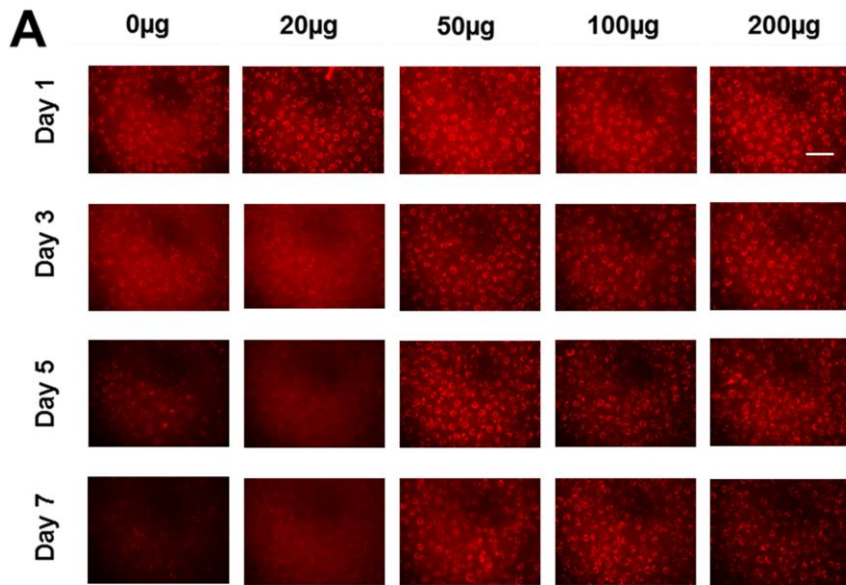


Figure 4. Dose of L-Histidine required to promote GET-peptide activity. Effect of different quantities of L-Histidine on P21mR8R stability upon storage in cell culture media of pH 3 (pH was adjusted using 1M acetic acid solution). 200µg/ml P21mR8R solution was prepared with various quantities L-Histidine (0 – 200µg) and stored at 37°C. A sample was taken from each solution, at defined time points, and diluted 1:10 in fresh cell culture media. Protein was then transduced into cells by addition to plated NIH3t3 cells. (A) Fluorescence microscopy images of the NIH3t3 cells treated with the sampled P21mR8R (Scale bar 10µm). (B) Flow cytometry histograms showing cellular uptake of P21mR8R after application of the samples to NIH3t3 cells. The red histograms represent P21mR8R samples taken at day 1, while the grey histograms represent day 7 samples, black histograms are the experiment control. (C) Relative transduction activity of the sampled P21mR8R. Protein transduction activity was compared to the experiment control. Error bars indicate SD (n = 3). * is p<0.05.

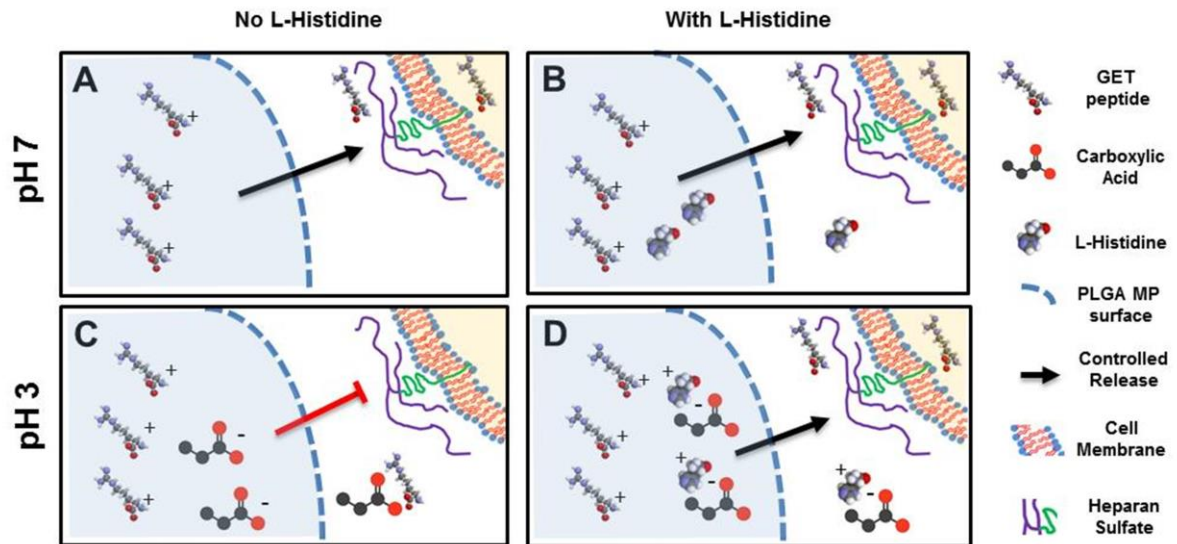


Figure 5. Schematic for hypothesis of GET peptides interaction with Carboxyl groups and L-Histidine. Interaction between GET peptides released from MPs and cells in presence of carboxyl groups resulted from PLGA degradation, with and without L-Histidine. (A) pH 7 without L-Histidine. (B) pH 7 with L-Histidine. (C) pH 3 without L-Histidine. (D) pH 3 with L-Histidine.

Supplemental Information:

Controlled release of GAG-binding Enhanced Transduction (GET) peptides for sustained and highly efficient intracellular delivery

Results

1. GET peptides activity is inhibited in presence of other acidic groups

In order to study the GET peptides stability in presence of other acidic groups, GET peptides were incubated, for 7 days, in media of various pH values (3, 5 & 7), the pH was adjusted using different solutions, 1M phosphoric acid (weak acid), 1M hydrochloric acid (HCl) (strong acid), or 1M sulfuric acid (strong acid). The experiment schematic is shown in figure S5 A. The collected samples were then applied to plated NIH 3t3 cells and cellular uptake of GET peptides was evaluated using fluorescence microscopy and flow cytometry. Fluorescence microscopy showed significant protein internalisation, for samples stored at pH 3, at day 1, for samples stored in media containing phosphoric acid. However, protein transduction was undetectable for day 7 samples (Figure S5 B). Flow cytometry results confirmed a loss in transduction for day 7 samples (Figure S5 B), and gradual loss of relative transduction activity over the time-points, reaching values below 20% at day 7 (Figure S5 D & F). Interestingly, fluorescent microscopy showed protein transduction for samples stored at pH 5 & 7 at all studied time points (Figure S5 B). Flow cytometry results confirmed this with relative transduction activity above 80% of non-treated controls at all studied time points. This behaviour is comparable to the previous obtained results using lactic, glycolic and acetic acid (section 3.8.1). On the other hand, fluorescent microscopy for samples stored at pH 3 media containing strong acids (hydrochloric or sulfuric acid) showed major loss of GET peptides

activity from day 1 (Figures S6 A & S7 A), with relative transduction activity below 40% (Figures S6 E and S7 E). Samples stored at pH 5 & 7 showed minimal loss of activity with relative transduction activity above 60% in all studied time points (Figures S6 E & S7 E).

2. L-Histidine protect GET peptides in presence of weak acid but not strong acid

To determine if L-Histidine could promote GET-protein transduction in presence of various acidic groups, we repeated the pH incubation experiments in the presence of L-Histidine. As before, protein was stored in cell culture media of various pH values (3, 5 & 7); adjusted using either 1M phosphoric acid, 1M hydrochloric acid (HCl), or 1M sulfuric acid. In presence of phosphoric acid (weak acid) L-Histidine (200µg/ml) containing samples (either for fluorescence microscopy and flow cytometry analyses) showed comparable P21mR8R transduction activity for all the studied pH conditions including those at pH 3 (Figure S5 C, E & G). Protein relative transduction activity was above 60% for all the studied conditions (Figure S5 G) and therefore it was clear that the presence of L-Histidine during incubation prevented the loss of GET transduction activity. On the other hand, L-Histidine did not protect GET peptides at pH 3 in presence of strong acids (HCl or sulfuric acid) (Figures S6 B & F and S7 B & F), with relative transduction activity below 40% from day 1.

3. L-Histidine does not neutralize carboxyl group acidity

In order to clarify why L-Histidine has such profound effect on carboxyl acid-mediated inhibition of GET transduction, we determined if the addition of L-Histidine could neutralize the acidity of the carboxyl acid-containing media. The pH of the media after addition of L-Histidine (200µg/ml) was measured in the three studied pH conditions (3, 5 & 7). The pH found to be the same, with no significant change

(Figure S8). Also, to confirm the quantities of L-Histidine required to neutralize the pH we employed, we performed a back titration with L-Histidine. Our titration showed that the molar ratio of 1:5 between the acetic acid and L-Histidine is required for neutrality. Accordingly, it is very unlikely that the L-Histidine is neutralizing the pH conditions in our system, but will work as a scavenger competing with PLGA degradation products for GET peptide interaction.

4. Co-incubation with L-Histidine is required to prevent inhibition of GET transduction by carboxylic acid

In order to assess if the mechanism of L-Histidine was during incubation, or could be manifest by its addition post-incubation, we repeated the pH incubation experiments. After samples were taken and diluted, 20 μ g L-Histidine was added and transduction activity tested. Fluorescent microscopy showed no protein transduction in the cells incubated at pH 3 with or without addition of L-Histidine post-incubation (Figure S9A). Flow cytometry confirmed that L-Histidine addition had no effect unless it was present throughout the pH incubation (Figure S9 B), with relative transduction activity around ~20%. It is therefore likely that L-Histidine prevents P21mR8R/carboxylic acid interaction or pH-dependent inhibition of transduction which is a gradual process over the 7 days experiment.

5. Poly-L-Histidine also prevents the inhibition of GET transduction in carboxylic acid/low pH conditions

In order to confirm that other histidine containing molecules can also help prevent the inhibition of GET transduction activity from PLGA-MPs release, we assessed if Poly-L-Histidine can also mediate this effect. We repeated the pH stability assay with the addition of Poly-L-Histidine (200 μ g/ml). Fluorescence microscopy showed protein

internalization in the cells for all pH conditions at all time points containing Poly-L-Histidine versus a loss of transduction with no excipient (Figure S10 B). Flow cytometry data showed gradual loss of protein transduction activity at pH 7 (Figure S10 C), with relative transduction activity of around 30% after 7 days (Figure S10 D). Protein transduction activity at pH 3 was above 50% at all time points (Figure S10 D). Protein incubated at pH 5 possessed transduction activity comparable to the experimental control (Figure S10 C), with relative transduction activity above 80% at all time points. This data therefore concludes that any L-Histidine containing excipient may be used to prevent the negative effect of carboxylic acid-mediated low pH on transduction.

Supplemental Figures:

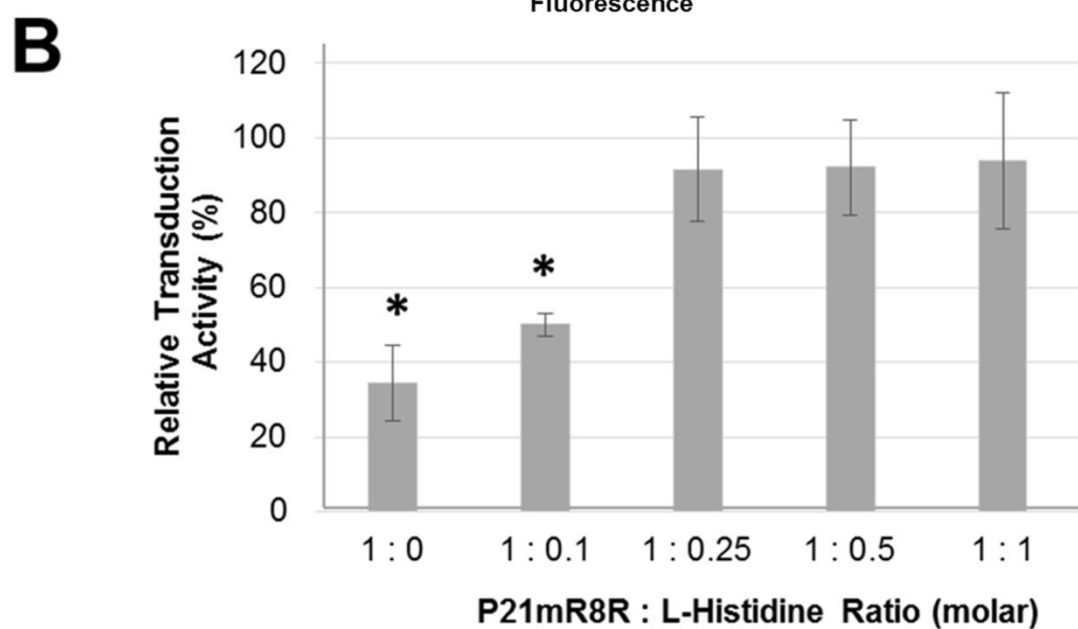
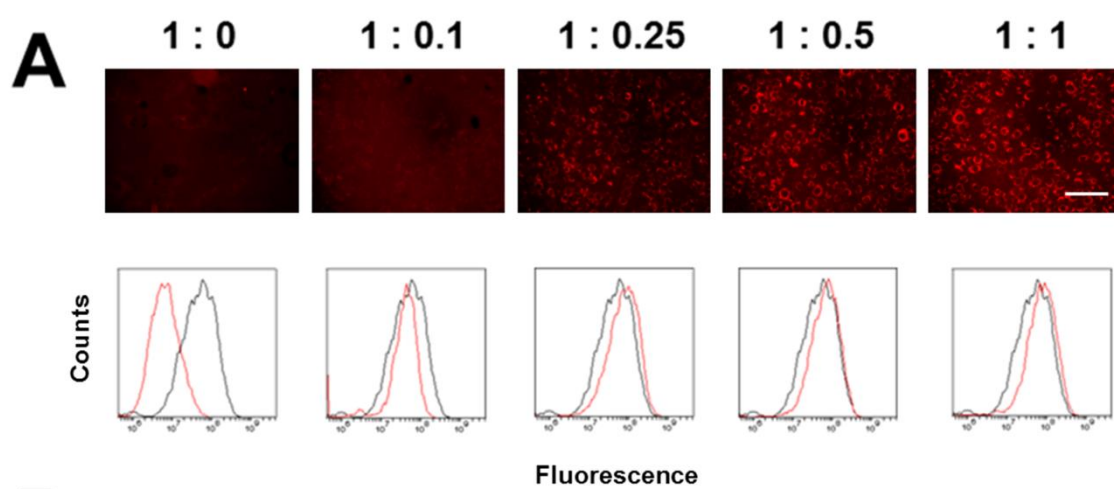


Figure S1. Effect of L-Histidine on GET peptides stability in media contains PLGA degradation product. Blank PLGA MPs were left to degrade in media, after that P21mR8R was mixed with the media and different quantities of L-Histidine. Protein was then transduced into cells by addition to plated NIH3t3 cells. (A) Fluorescence microscopy images of the NIH3t3 cells treated with the sampled P21mR8R (Scale bar 10 μ m) and flow cytometry histograms showing cellular uptake of P21mR8R after application of the samples to NIH3t3 cells. The red histograms represent P21mR8R samples while black histograms are the experiment control. (B) Relative transduction activity of the sampled P21mR8R. Protein transduction activity was compared to the experiment control. Error bars indicate SD (n = 3). * is p<0.01.

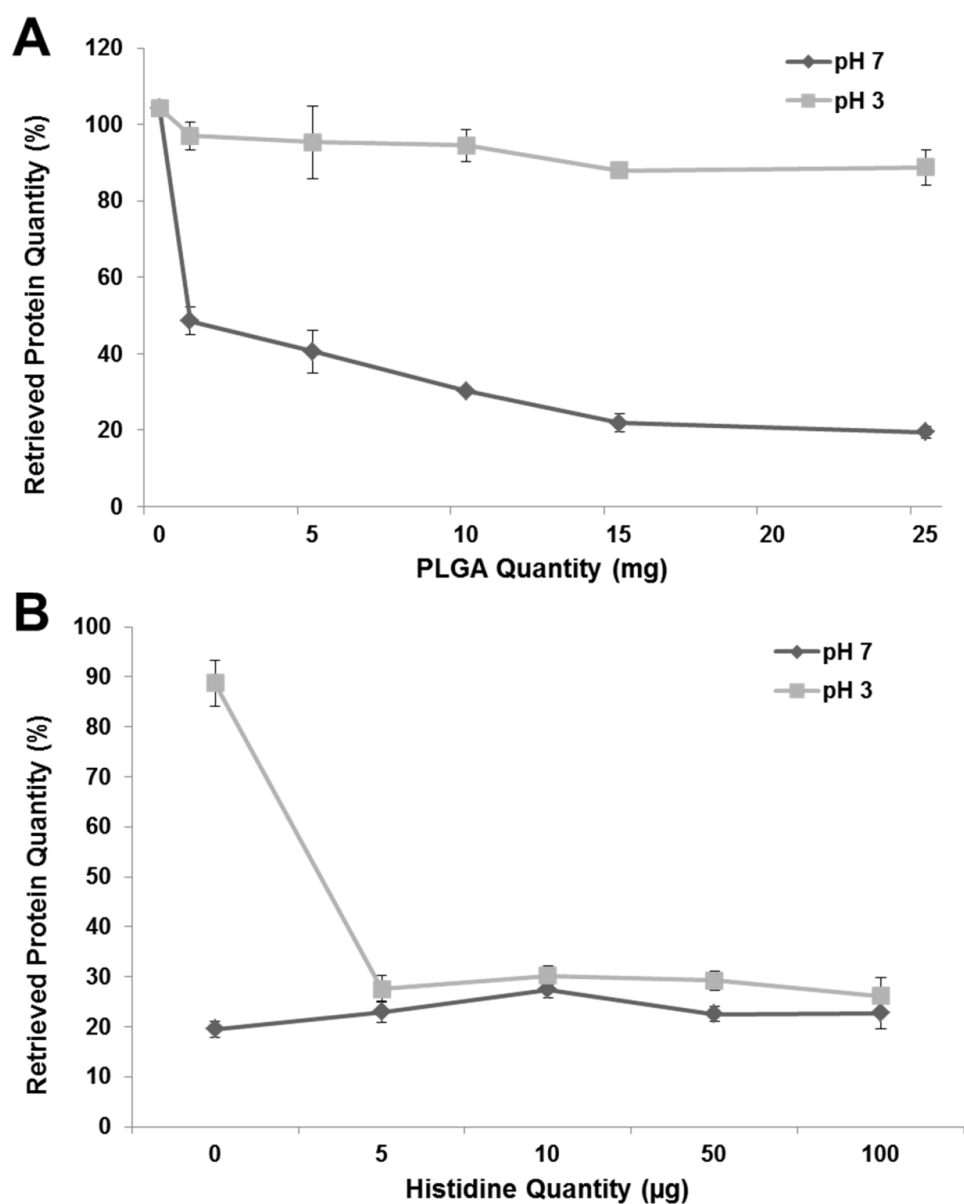


Figure S2. GET peptides interaction with blank PLGA MPs at different pH conditions with and without L-Histidine. PLGA MPs were suspended in solution of different pH values and P21mR8R was then added, the solution was then centrifuged and the P21mR8R quantity in the supernatant was measured. (A) P21mR8R interaction with various blank PLGA MPs quantity at various pH conditions without L-Histidine. (B) P21mR8R interaction with 25mg PLGA MPs at various pH conditions in presence of different L-Histidine quantities. Error bars indicate SD (n = 3).

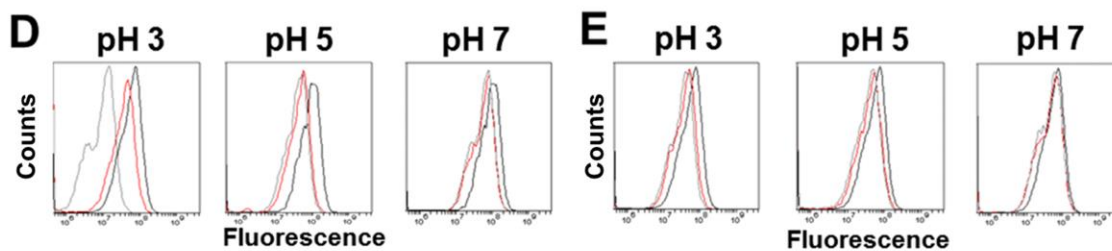
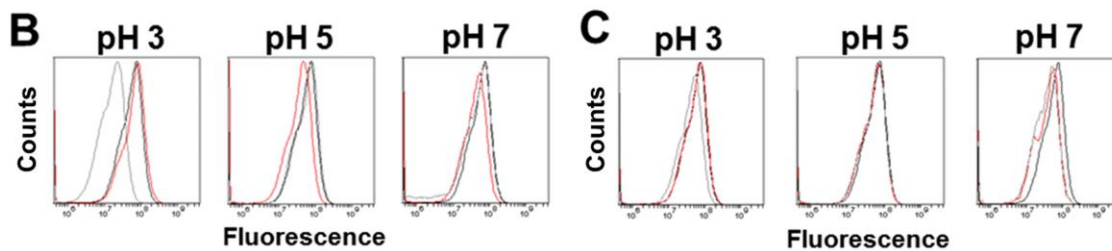
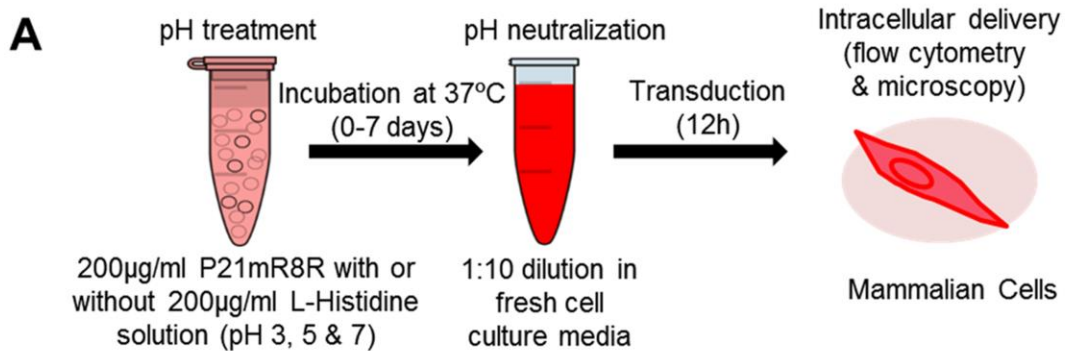


Figure S3. GET-peptide stability at various pH values mimicking the PLGA microenvironment. P21mR8R stability in cell culture media of various pH values (3, 5 & 7), with and without L-Histidine. pH was adjusted using either 1M lactic acid solution or 1M glycolic acid. (A) Schematic of the protein storage and sampling, 200µg/ml P21mR8R with or without 200µg/ml L-Histidine solution was prepared with various pH values (3, 5 & 7) and stored at 37°C. A sample was taken from each solution, at defined time points, and diluted 1:10 in fresh cell culture media. Protein was then transduced into cells by addition to plated NIH3t3 cells. Flow cytometry histograms showing cellular uptake of P21mR8R after application of the samples to NIH3t3 cells (B) samples contain P21mR8R and pH adjusted using 1M lactic acid solution (C) samples contain P21mR8R and L-Histidine and pH adjusted using 1M lactic acid solution (D) samples contain P21mR8R and pH adjusted using 1M glycolic acid solution (E) samples contain P21mR8R and L-Histidine and pH adjusted using 1M glycolic acid solution. The red histograms represent P21mR8R samples taken at day 1, while the grey histograms represent day 7 samples, black histograms are the experiment control.

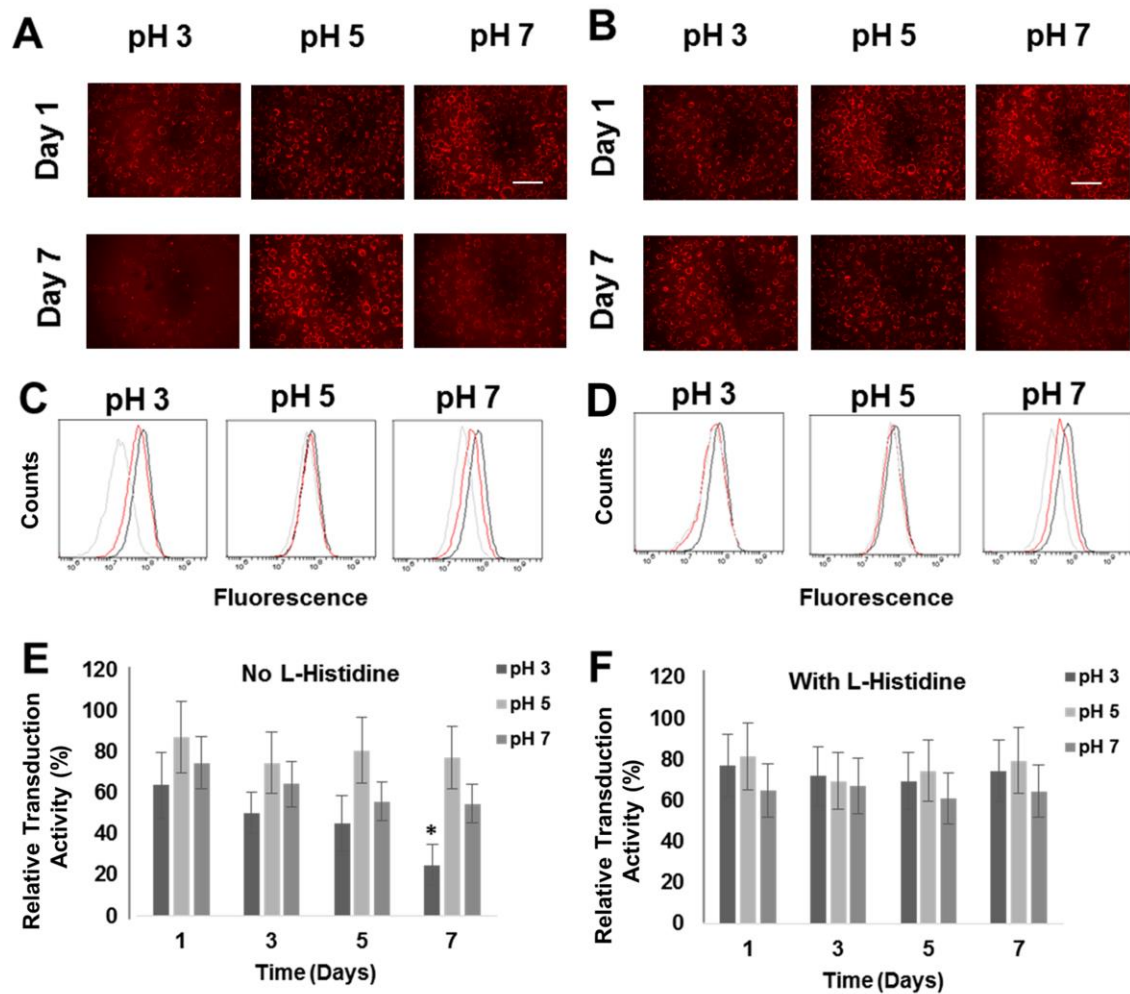


Figure S4. GET-peptide stability at various pH values mimicking the PLGA microenvironment. P21mR8R stability in cell culture media of various pH values (3, 5 & 7), with and without L-Histidine. pH was adjusted using 1M acetic acid solution. A sample was taken from each solution, at defined time points, and diluted 1:10 in fresh cell culture media. Protein was then transduced into cells by addition to plated NIH3t3 cells. (A) Fluorescence microscopy images of the NIH3t3 cells treated with the samples contain P21mR8R (Scale bar 10 μ m). (B) Fluorescence microscopy images of the NIH3t3 cells treated with the samples contain P21mR8R and L-Histidine (Scale bar 10 μ m). Flow cytometry histograms showing cellular uptake of P21mR8R after application of the samples to NIH3t3 cells (C) samples contain P21mR8R (D) samples contain P21mR8R and L-Histidine. The red histograms represent P21mR8R samples taken at day 1, while the grey histograms represent day 7 samples, black histograms are the experiment control. Relative transduction activity of the P21mR8R (E) samples contain P21mR8R (F) samples contain P21mR8R and L-Histidine. Protein transduction activity was compared to the experiment control. Error bars indicate SD (n = 3). * is p<0.05.

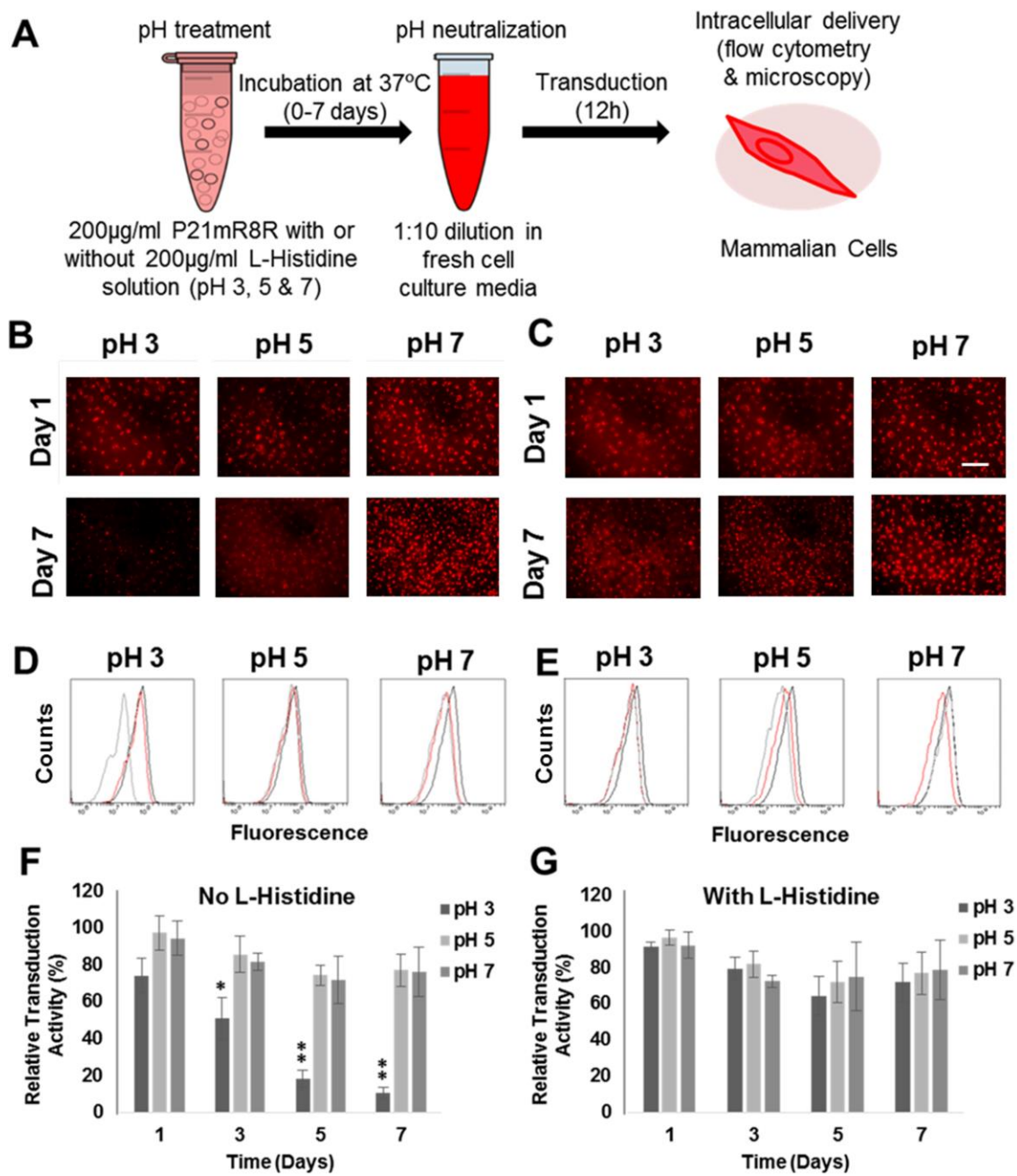


Figure S5. GET-peptide stability at various pH values mimicking the PLGA microenvironment. P21mR8R stability in cell culture media of various pH values (3, 5 & 7), with and without L-Histidine. pH was adjusted using 1M phosphoric acid solution. (A) Schematic of the protein storage and sampling, 200µg/ml P21mR8R with or without 200µg/ml L-Histidine solution was prepared with various pH values (3, 5 & 7) and stored at 37°C. A sample was taken from each solution, at defined time points, and diluted 1:10 in fresh cell culture media. Protein was then transduced into cells by addition to plated NIH3t3 cells. (B) Fluorescence microscopy images of the NIH3t3 cells treated with the samples contain P21mR8R (Scale bar 10µm). (C) Fluorescence microscopy images of the NIH3t3 cells treated with the samples contain P21mR8R and L-Histidine (Scale bar 10µm). Flow cytometry histograms showing cellular uptake of P21mR8R after application of the samples to NIH3t3 cells (D) samples contain P21mR8R (E) samples contain P21mR8R and L-Histidine. The red histograms represent P21mR8R samples taken at day 1, while the grey histograms represent day 7 samples, black histograms are the experiment control. Relative biological activity of the P21mR8R (F) samples contain P21mR8R (G) samples contain P21mR8R and L-Histidine. Protein transduction activity was compared to the experiment control. Error bars indicate SD (n = 3). * is p<0.05, ** is p<0.01.

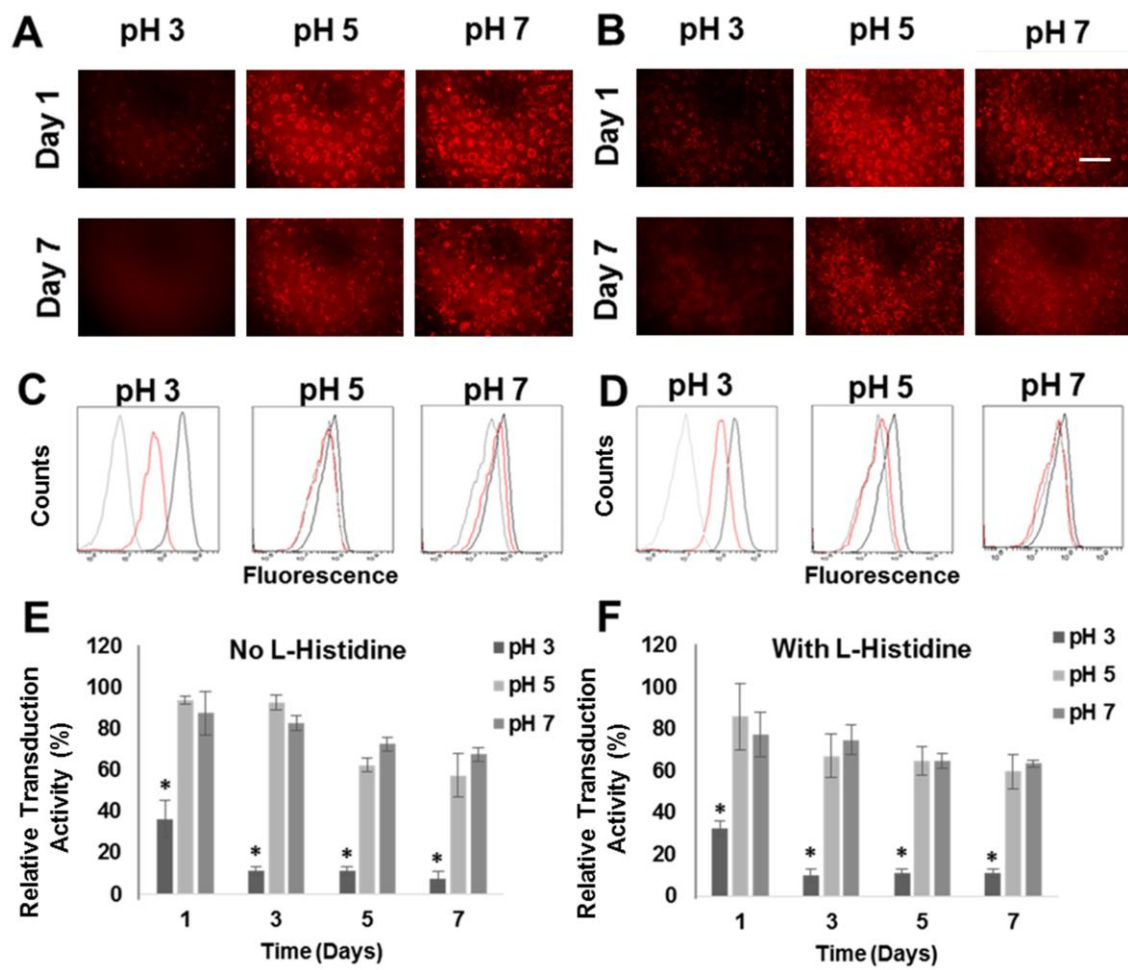


Figure S6. GET-peptide stability at various pH values mimicking the PLGA microenvironment. P21mR8R stability in cell culture media of various pH values (3, 5 & 7), with and without L-Histidine. pH was adjusted using 1M hydrochloric acid solution. A sample was taken from each solution, at defined time points, and diluted 1:10 in fresh cell culture media. Protein was then transduced into cells by addition to plated NIH3t3 cells. (A) Fluorescence microscopy images of the NIH3t3 cells treated with the samples contain P21mR8R (Scale bar 10 μ m). (B) Fluorescence microscopy images of the NIH3t3 cells treated with the samples contain P21mR8R and L-Histidine (Scale bar 10 μ m). Flow cytometry histograms showing cellular uptake of P21mR8R after application of the samples to NIH3t3 cells. (C) Samples contain P21mR8R (D) samples contain P21mR8R and L-Histidine. The red histograms represent P21mR8R samples taken at day 1, while the grey histograms represent day 7 samples, black histograms are the experiment control. Relative transduction activity of the P21mR8R (E) samples contain P21mR8R (F) samples contain P21mR8R and L-Histidine. Protein transduction activity was compared to the experiment control. Error bars indicate SD (n = 3). * is p<0.05, ** is p<0.01.

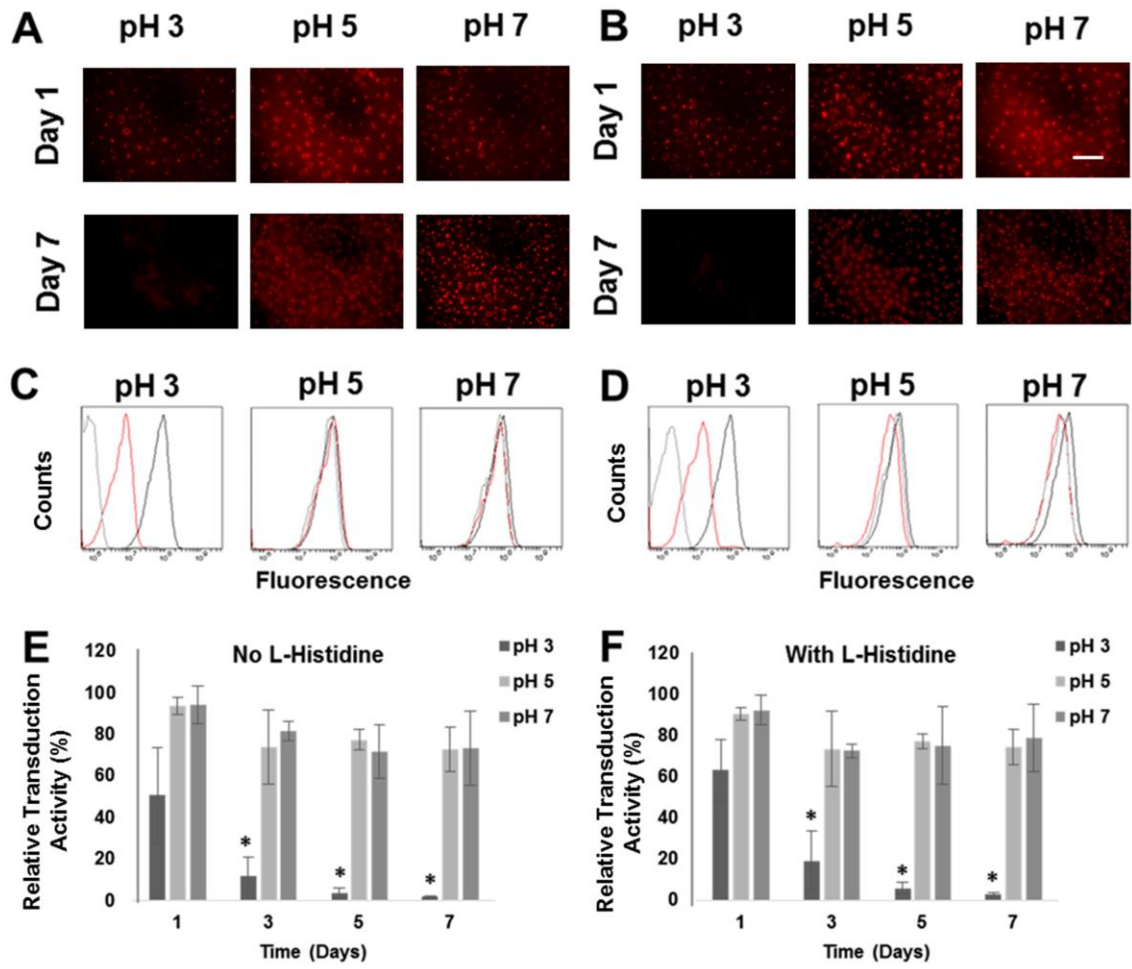


Figure S7. GET-peptide stability at various pH values mimicking the PLGA microenvironment. P21mR8R stability in cell culture media of various pH values (3, 5 & 7), with and without L-Histidine. pH was adjusted using 1M sulfuric acid solution. A sample was taken from each solution, at defined time points, and diluted 1:10 in fresh cell culture media. Protein was then transduced into cells by addition to plated NIH3t3 cells. (A) Fluorescence microscopy images of the NIH3t3 cells treated with the samples contain P21mR8R (Scale bar 10 μ m). (B) Fluorescence microscopy images of the NIH3t3 cells treated with the samples contain P21mR8R and L-Histidine (Scale bar 10 μ m). Flow cytometry histograms showing cellular uptake of P21mR8R after application of the samples to NIH3t3 cells (C) samples contain P21mR8R (D) samples contain P21mR8R and L-Histidine. The red histograms represent P21mR8R samples taken at day 1, while the grey histograms represent day 7 samples, black histograms are the experiment control. Relative transduction activity of the P21mR8R (E) samples contain P21mR8R (F) samples contain P21mR8R and L-Histidine. Protein transduction activity was compared to the experiment control. Error bars indicate SD (n = 3). * is p<0.05.

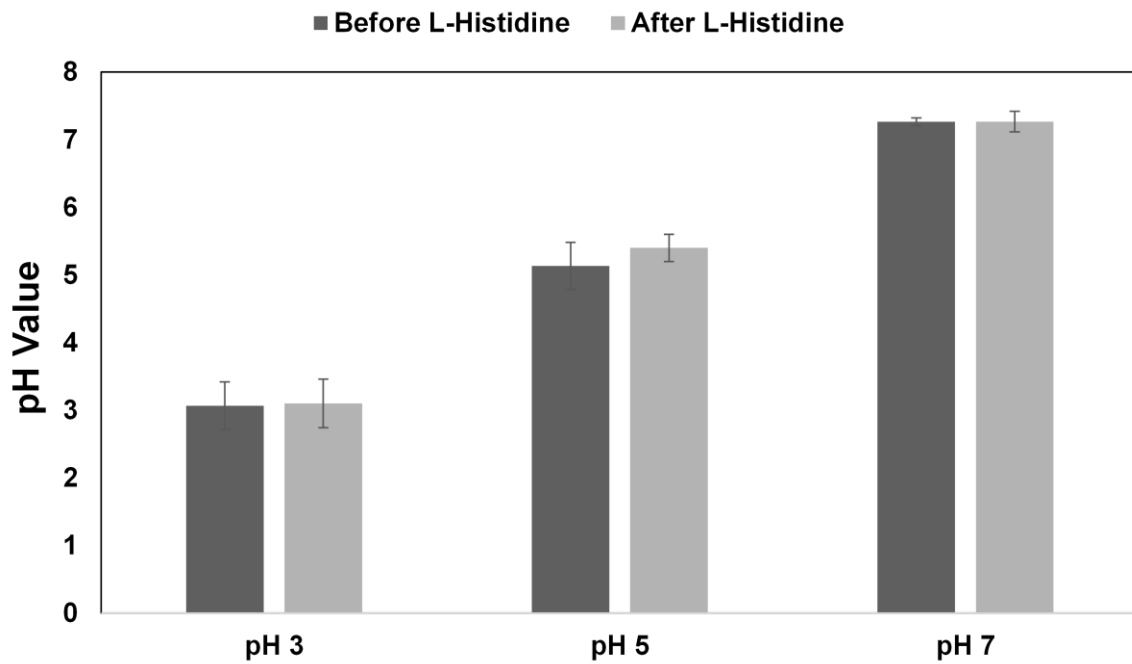


Figure S8. L-Histidine does not change pH values. pH values for media before and after addition of L-Histidine (200 μ g/ml). pH was initially adjusted using 1M acetic acid solution. Error bars indicate SD (n = 3).

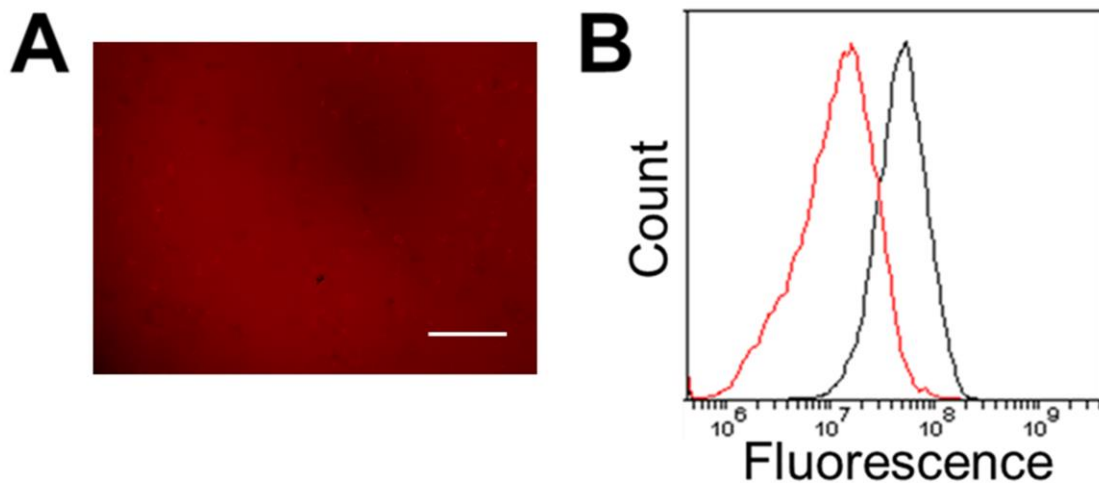


Figure S9. L-Histidine promotes GET-peptide activity. Effect of L-Histidine on P21mR8R stability upon storage in cell culture media of pH 3, adjusted using 1M acetic acid solution. 200 μ g/ml P21mR8R solution of pH 3 was prepared and stored at 37°C for 7 days. A sample was taken from the solution, after 7 days, and diluted 1:10 in fresh cell culture media, producing a P21mR8R solution of 20 μ g/ml. 20 μ g of L-Histidine was then added to the solution. Protein was then transduced into cells by addition to plated NIH3t3 cells. (A) Fluorescence microscopy images of the NIH3t3 cells treated with the sampled P21mR8R (Scale bar 10 μ m). (B) Flow cytometry histograms showing cellular uptake of P21mR8R after application of the sample to NIH3t3 cells. The red histograms represent P21mR8R sample, black histogram is the experiment control.

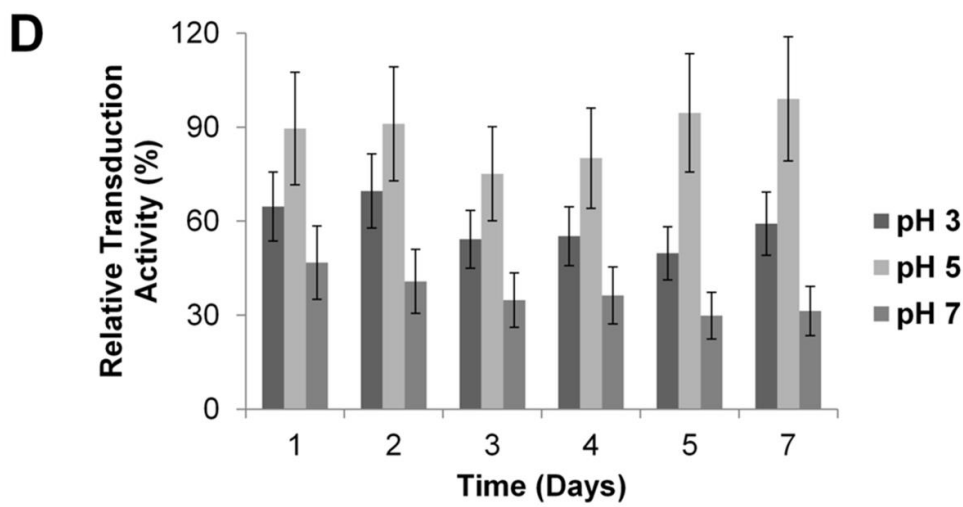
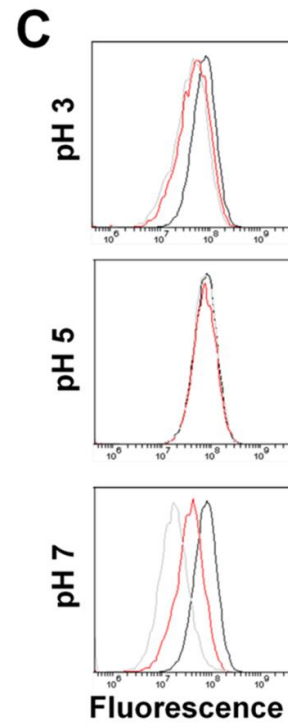
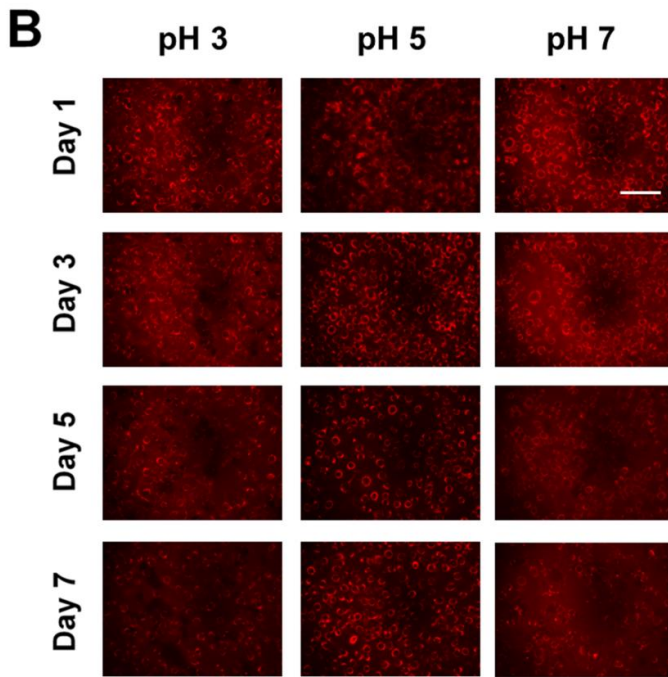
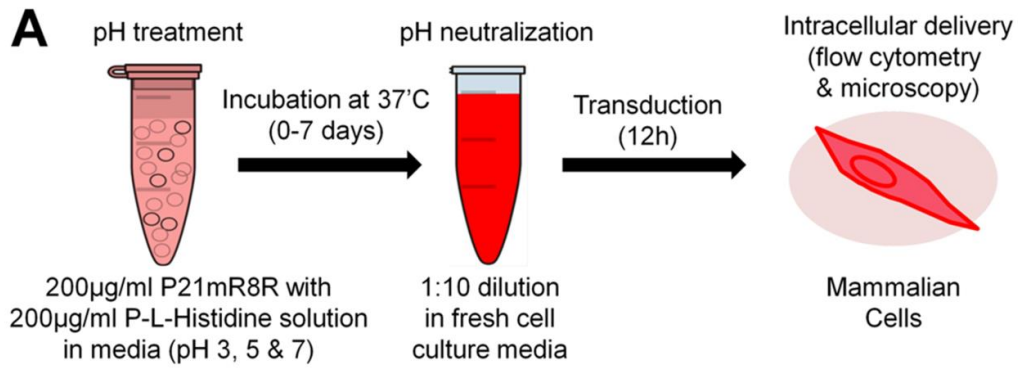


Figure S10. Poly-L-Histidine promotes GET-peptide activity. Effect of Poly-L-Histidine on P21mR8R stability upon storage in cell culture media of various pH values (3, 5 & 7), adjusted using 1M acetic acid solution. (A) Schematic of the protein storage and sampling, 200 μ g/ml P21mR8R and 200 μ g/ml Poly-L-Histidine solution was prepared with various pH values (3, 5 & 7) and stored at 37°C. A sample was taken from each solution, at defined time points, and diluted 1:10 in fresh cell culture media. Protein was then transduced into cells by addition to plated NIH3t3 cells. (B) Fluorescence microscopy images of the NIH3t3 cells treated with the sampled P21mR8R (Scale bar 10 μ m). (C) Flow cytometry histograms showing cellular uptake of P21mR8R after application of the samples to NIH3t3 cells. The red histograms represent P21mR8R samples taken at day 1, while the grey histograms represent day 7 samples, black histograms are the experiment control. (D) Relative transduction activity of the sampled P21mR8R. Protein transduction activity was compared to the experiment control. Error bars indicate SD (n = 3).

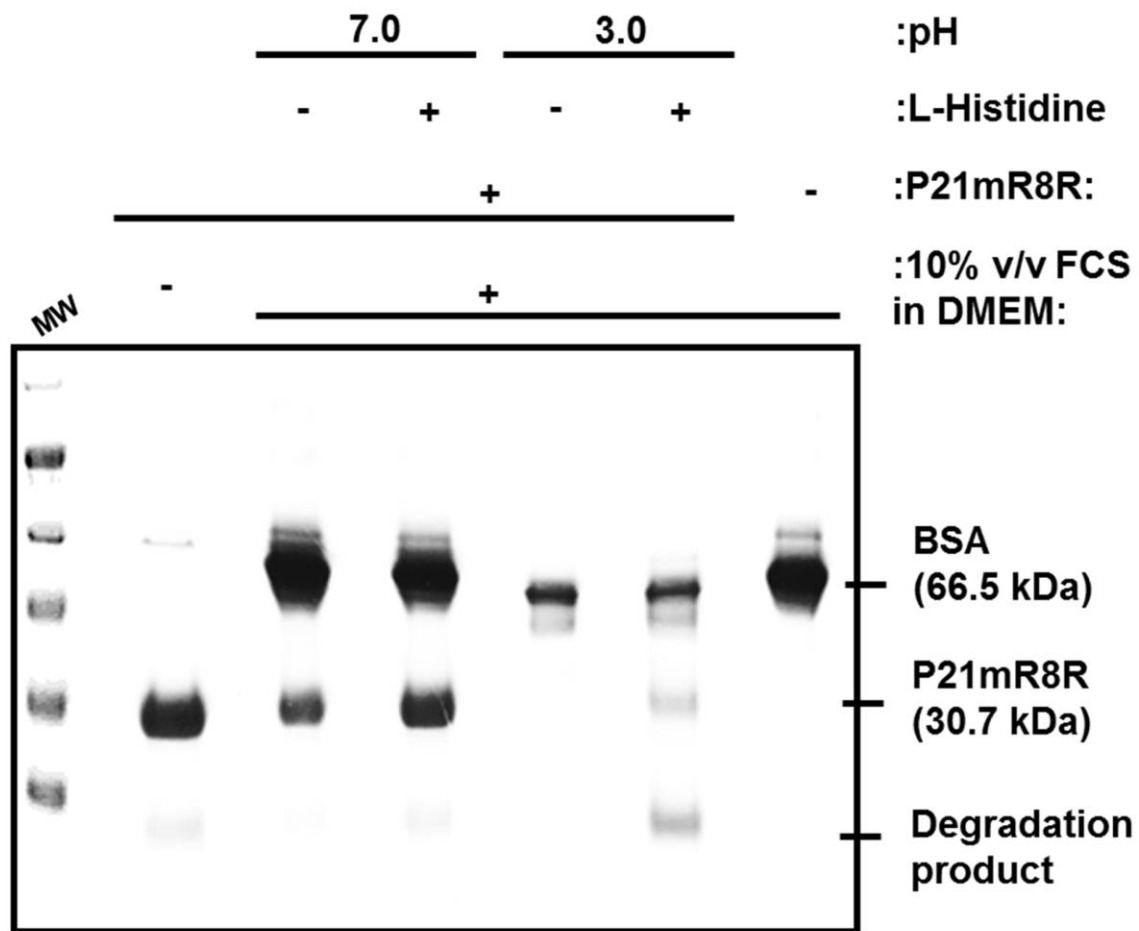


Figure S11. Estimation of P21mR8R molecular weight using SDS-PAGE.

P21mR8R was incubated for 7 days in media of various pH conditions (3 & 7) with and without L-histidine. P21mR8R band was then compared to fresh protein sample. DMEM media containing FCS was used as a control.

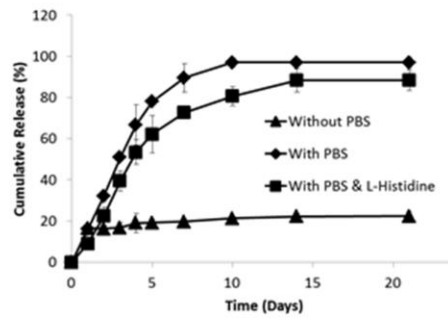
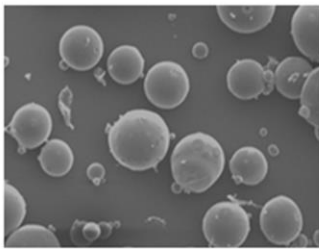
pH	ZP (mV) Average (\pm SD)
3.0	- 15.6 (1.3)
5.0	-7.9 (1.0)
7.0	-1.0 (0.4)

Table S1. Blank MPs surface charge at various pH conditions (3, 5 & 7)

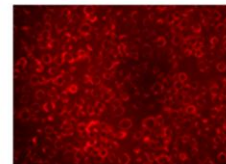
Peptide	Molecular Weight (kDa)	Isoelectric point (pI)	Charge at various pH										
			3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
mR	26.6	5.7	36.1	33.1	26.6	16.4	7.3	2.3	-0.3	-2.2	-3.8	-4.9	-5.7
P21	2.5	10.9	12.5	12.2	11.8	11.5	11.2	11.1	11	11	10.9	10.6	10.2
8R	1.3	12.9	8.6	8.3	8.1	8	8	8	8	8	7.9	7.8	7.5
P21mR8R	30.7	9.6	56.1	53	46.3	35.8	26.5	21.3	18.7	16.7	15.1	14	13

Table S2. P21mR8R, mR, P21 & 8R theoretical molecular weights, isoelectric points and charge at various pH values.

1. *In vitro* Protein release from microparticles

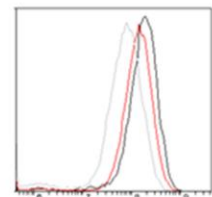
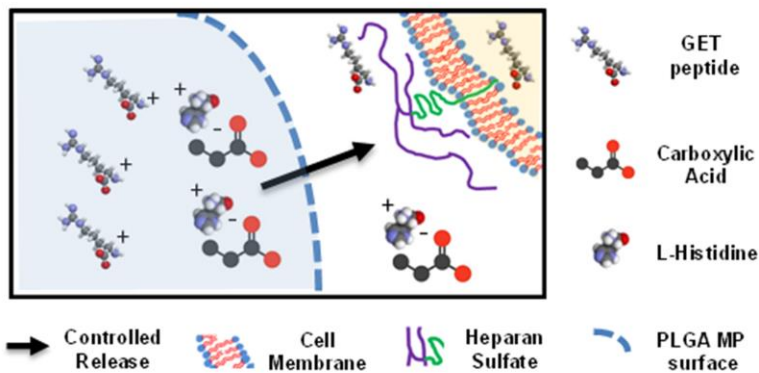


2. GET protein intracellular transduction



Microscopy

3. L-Histidine enhancement of released GET transduction



Flow cytometry

Graphical Abstract