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A pH-sensitive eosin-block copolymer delivers proteins intracellularly

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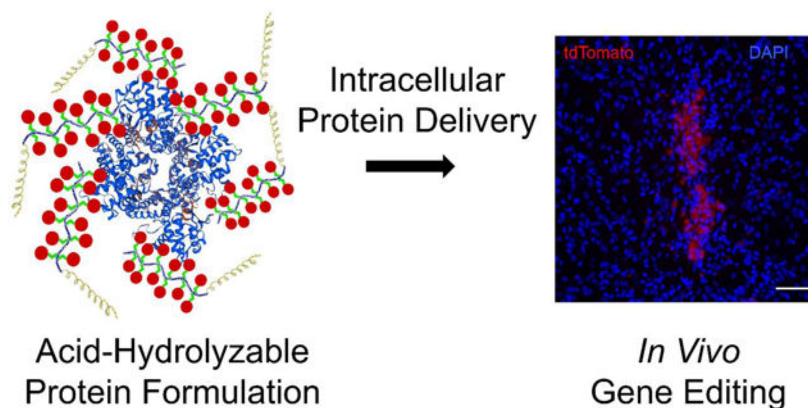
Abstract

There is great interest in developing strategies to deliver proteins into the cytoplasm of cells. We report here a PEG-poly-eosin block copolymer (PEG-pEosin) that can encapsulate proteins and release them in active form under mildly acidic conditions. A PEG-pEosin formulation composed of Cre and the endosomomolytic protein LLO efficiently performed gene editing in cells and in the brains of mice after an intracranial injection.

Graphical Abstract



An acid-hydrolyzable PEG-poly-eosin polymer (PEG-pEosin) complexes and shields proteins reversibly and delivers proteins intracellularly. *In vivo* gene editing in mouse brains is shown

Protein therapeutics have the potential to revolutionize medicine and are currently being investigated for the treatment of numerous diseases.[1] Since the US Food and Drug

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Conflicts of interest
There are no conflicts to declare

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Administration (FDA) approved the first recombinant protein therapeutic in 1982, more than 200 proteins or peptides have been approved by the FDA.[2] Recently approved protein therapeutics address a wide variety of clinical indications, including cancers, autoimmunity/inflammation, exposure to infectious agents, and genetic disorders.[3]

Current protein therapeutics exclusively act on extracellular targets. However, there are a large number of diseases that could be treated by delivering proteins intracellularly, and there is consequently great interest in developing new delivery vehicles that can deliver proteins intracellularly.[4] Several strategies have been developed to deliver proteins intracellularly, such as, cationic oligomer and polymer formulations,[5, 6] lipid nanoparticles,[7, 8] nanocapsules,[9] polymersomes,[10, 11] liposomes,[12] nanogels,[13] and polymer micelles.[14]

However, developing effective intracellular protein delivery vehicles has been challenging, due to the low charge density of proteins and their sensitivity to denaturation. For example, polycationic materials, such as solid lipid nanoparticle (SLNs) and polycations, can efficiently deliver nucleic acids *in vivo* and even in human patients, whereas protein delivery vehicles have had limited success *in vivo*. A key factor that has allowed nucleic acid delivery vehicles to rapidly progress is the strong electrostatic binding between cationic materials and negatively charged nucleic acids. However, no analogous complexation strategy for proteins exists. There is consequently a great need for the development of new protein complexation strategies that can allow delivery vehicles to bind proteins reversibly, without causing denaturation.

In this report, we demonstrate that the protein binding dye eosin can be used as a scaffold for developing delivery vehicles that can complex proteins reversibly. Eosin is a protein binding dye, that binds a wide number of proteins with a Kd between 10^{-3}-10^{-6} M, due to a combination of hydrophobic and electrostatic forces, and is currently used as a dye for staining proteins in gels. Eosin therefore has potential as a fragment for developing delivery vehicles, however, eosin has an off-rate from proteins that is under 1 second, and binds proteins too weakly to act as an effective delivery vehicle.

We hypothesized that a multivalent eosin, termed PEG-pEosin, generated via conjugation of eosin to a polymer backbone, would be able to bind proteins with the affinity needed to develop new protein delivery vehicles. Multivalent interactions are essential in the case of polycation-nucleic acid interactions, individual cations bind nucleic acids with very weak affinity (Kd approx. 1–10 M), but polycations can bind nucleic acids with Kds<<1 nM, due to the effects of multivalency. A multivalent eosin should similarly have a high binding constant with proteins, and should have a Kd that is orders of magnitude higher than monomeric eosin, due to cooperative binding of the eosins with the protein. In addition, we engineered acid-degradable linkages between the eosin and the polymer backbone to ensure that the protein is released from the polymer backbone after endocytosis.

The chemical structure of PEG-pEosin is shown in Figure 1. PEG-pEosin is a block copolymer composed of a poly(ethylene glycol)-b-poly(α,β-aspartic acid) that has multiple eosins conjugated to its aspartic acid segment, via a pH-sensitive ketal linker. PEG-pEosin

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should form core-shell block copolymers with proteins, due to the interactions between the poly-eosin and the protein, and their sizes should be limited by the presence of the PEG chain, which should form the corona of the micelles.

After endocytosis, the eosins will be hydrolyzed from the PEG-poly-eosin block copolymer and the protein will be released from the polymer. In this report, we show that PEG-pEosin was able to complex proteins with varying sizes and isoelectric points, in particular bovine serum albumin (BSA), the gene-editing enzyme Cre, and the endosomal-disruptive protein LLO. In addition, we demonstrate that a PEG-pEosin formulation that encapsulated Cre and LLO was able to dramatically increase the intracellular delivery of Cre, and also lower the toxicity of LLO. These results demonstrate that the eosin fragment has great potential as a scaffold for developing protein delivery vehicles.

The synthesis of PEG-pEosin is shown in Figure S1. A two-step synthetic route was used to generate PEG-pEosin. A PEG-pAsp(OBn) polymer with benzyl groups on the aspartic acid side chains was reacted with a diamine ketal linker, generating PEG-pAsp(ketal). PEG-pAsp(ketal) was reacted with eosin 5-isothiocyanate to generate PEG-pEosin, the final product was purified by precipitation in cold n-hexane/ether 50/50 and dialysis against a 7kDa MWCO spin column. NMR analysis of PEG-Eosin demonstrated that >90% of the amines of PEG-pAsp(ketal) had been modified with eosin 5-isothiocyanate.

PEG-pEosin is designed to complex proteins reversibly. We therefore investigated if PEG-pEosin could complex the protein BSA. BSA was used as a model protein for binding studies because of its well-studied interactions with eosin. BSA was mixed with PEG-pEosin at different molar ratios in PBS at pH 7.4 and the protein binding ability was determined by measuring their electrophoretic mobility in a 10% native PAGE gel. As a control, eosin 5-isothiocyanate was also mixed with BSA and analyzed by gel electrophoresis. Gel electrophoresis is a particularly challenging environment for a protein complex to form, because transient dissociation of the complex will result in a permanent loss of binding, due to the separation of the protein and polymer in the electric field.

Figure 2 demonstrates that PEG-pEosin can bind BSA. For example, the BSA protein band is completely eliminated after incubation with PEG-pEosin at a 6:1 molar ratio. In contrast, incubation of BSA with eosin by itself had no effect on the mobility of BSA. The results of Figure 2A suggest that PEG-pEosin is forming high molecular weight complexes with BSA, which cannot migrate through the gel. To further verify that PEG-pEosin was complexing BSA, DLS was performed on the BSA+PEG-pEosin solutions. Figure S2 demonstrates that BSA+PEG-pEosin has a bimodal distribution, with an average diameter of 24 nm. Figure S3 verifies that free BSA is complexed in larger nanoparticles between 10 – 30 nm. The small size of the formulation indicates, that single BSA molecules are probably complexed with PEG-pEosin. The minor second peak in the size distribution of BSA + PEG-pEosin overlaps with PEG-pEosin alone, which can be explained by the presence of free PEG-pEosin, due to its 6-fold molar excess over BSA.

PEG-pEosin is designed to release proteins under acidic conditions, due to the hydrolysis of its ketal linkage and the release of eosin. The ketal linkage in PEG-pEosin has a half-life of

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approximately 4 hours at pH 7.4 and 24 minutes at pH 6.4, however this will most likely be slower after conjugation to the PEG-pEosin backbone. The pH-dependent release of BSA from PEG-pEosin at the pH values of 6.8, 6.4, 5.5 or 5.0, was investigated. Figure 2B demonstrates that PEG-pEosin binds BSA in a pH-dependent manner. For example, PEG-pEosin partially releases BSA at pH 6.8, but completely releases it at pH 6.0 and below. Thus, the binding of PEG-Eosin to BSA is mediated by multivalent eosin interactions, which are reduced after acid hydrolysis of the eosins from the polymer backbone. DLS analysis was also performed on the BSA+PEG-pEosin complex at acidic pH. Figure S2 demonstrates that at acidic pH a large-sized aggregated complex between BSA and PEG-pAsp is formed. Interestingly, although the hydrolysed PEG-pEosin can complex BSA by DLS, it is unable to form a complex that is stable enough to survive gel electrophoresis.

PEG-pEosin has the potential to bind a wide variety of proteins, given the broad classes of proteins that eosin binds. We investigated if PEG-pEosin could complex the protein lysteriolysin (LLO). LLO is a bacterial pore-forming toxin, secreted by Lysteria monocytogenes, which triggers the lysis of cell membranes with exceptional efficiency, and is also activated by acidic pH. investigated if PEG-pEosin could complex the protein lysteriolysin (LLO). LLO is a bacterial pore-forming toxin, secreted by Lysteria monocytogenes, which triggers the lysis of cell membranes with exceptional efficiency, and is also activated by acidic pH. LLO has consequently been investigated as an endosomal disruptive agent, however its toxicity has limited its potential uses. We therefore investigated if PEG-pEosin could complex LLO and prevent it from disrupting cell membranes at pH 7.4. An LLO+PEG-pEosin formulation would have numerous applications in drug delivery, such as for the intracellular delivery of co-formulated therapeutic proteins, and for targeting the acidic environment of tumors.

The pH-dependent membrane-disruptive properties of LLO+PEG-pEosin was determined using the hemolysis of red blood cell membranes as a surrogate marker for membrane disruption. LLO was mixed with PEG-pEosin, at various pH values, and the ability of the complex to disrupt membranes was investigated. LLO was used at 0.5 μg/mL concentration either free or mixed with 6 molar equivalents of PEG-pEosin. Figure 3 demonstrates that PEG-pEosin can reversibly inhibit the membrane-disruptive ability of LLO. For example, at pH 7.4, free LLO causes a 100% hemolysis of red blood cells after 30 minutes of incubation, whereas at pH 7.4, after 30 minutes, LLO+PEG-pEosin causes almost no membrane disruption. These results suggest that PEG-pEosin can complex LLO, via its eosin interactions and prevents LLO from inserting into cell membranes. Importantly, the membrane-disruptive activity of LLO is almost completely restored at pH 6.8 and pH 5.5, due to the hydrolysis of the ketal linkages connecting the eosin to PEG-pEosin. The recovery of LLO activity at acidic pHs demonstrates that complexing LLO with PEG-pEosin does not cause LLO denaturation.

We also measured the pH-dependent toxicity of LLO+PEG-pEosin on cells in culture to determine if it could act as an anticancer agent that could be selectively activated at the acidic pHs of the extracellular tumor environment. The cytotoxic effect of free and PEG-pEosin-formulated LLO on cancer cells was tested in HeLa cells at pH 7.4 and 6.8. Figure 4 demonstrates that LLO+PEG-pEosin is a pH-dependent cytotoxic formulation, which has

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potential as an anticancer agent. For example, at pH 7.4, a 2.5 μg/mL concentration of LLO, incubated for 4 hours, causes 80% toxicity to cells, whereas LLO+PEG-pEosin causes no toxicity, indicating that PEG-pEosin can complex LLO and shield it from cell membranes. At pH 6.8, LLO and LLO+PEG-pEosin have similar levels of toxicity because the LLO is released from the PEG-pEosin. Similarly, PEG-pEosin was also able to protect cells from LLO at pH 7.4 after a 24-hour incubation. In this case, the IC_{50} of free LLO was 1 μg/mL and the IC_{50} of LLO+PEG-pEosin was approximately 10 μg/mL. These results suggest that the LLO+PEG-pEosin complex is stable in the presence of biological media, and has potential as a platform for developing tumor-targeted therapeutics.

The LLO+PEG-pEosin formulation also has the potential to increase the intracellular delivery of proteins, given the ability of PEG-pEosin to complex a wide variety of proteins, and the efficient endosomal disruptive ability of LLO. We investigated if LLO+PEG-pEosin could enhance the intracellular delivery of Crerecombinase in 3T3 Ai9 cells. We selected Cre as a model enzyme for intracellular delivery because it provides an unambigous read-out of cytoplasmic delivery, as Cre has to be delivered into the nucleus for its effects to be measured in 3T3 Ai9 cells.

A co-formulation of Cre recombinase and LLO was made by adding 6 molar equivalents of PEG-pEosin to a solution of both proteins. DLS measurements of this formulation demonstrated that it had a z-average of 52.6 nm and a negative zeta potential of −8.2 mV, indicating that larger complexes with multiple proteins are formed compared to the smaller complexes of presumably single BSA molecules and PEG-pEosin (Figure S2). The gene editing efficiency of Cre+LLO+PEG-pEosin was tested in 3T3 Ai9 cells, where successful recombination results in expression of the fluorescent tdTomato protein. Formulations with 8 μg/mL (143 nM) and 12 μg/mL (214 nM) concentrations of LLO, 3.8 μg/mL (100 nM) Cre and 6 molar equivalents of PEG-pEosin (1.46 μM and 1.88 μM respectively) were tested. The gene editing efficiency was determined by flow cytometry (Figure 5A). Figure 5 demonstrates that LLO+PEG-pEosin can dramatically increase the delivery of Cre into cells. For example, Cre alone and Cre physically mixed with LLO showed no significant gene editing. In contrast, Cre and LLO mixed with PEG-pEosin showed gene-editing efficiencies of 12.3% with 8 μg/mL LLO and 20.5% with 12 μg/mL LLO. In addition, PEG-pEosin also reduced the toxicity of Cre+LLO (Figure 5B). Cre + LLO caused 80%−85% levels of cell toxicity, at 8 and 12 μg/mL, whereas the Cre+LLO+PEG-pEosin formulation had only 40–50% cell toxicity. These results suggest that some amount of LLO is being released from the micelles and indicate that additional work needs to be done with this formulation to enhance its stability. Cre+LLO+PEG-pEosin was lastly tested in the brains of Ai9 mice. Cre+LLO+PEG-pEosin was injected into the striatum of Ai9 mice and the gene editing efficiency was determined by detecting tdTomato+ cells. Figure 6 demonstrates that LLO+PEG-pEosin can efficiently deliver Cre after an intracranial injection, and demonstrate that Cre+LLO+PEG-pEosin can edit genes in the brain *in vivo*.

In conclusion, we present a novel pH-sensitive protein delivery vehicle PEG-pEosin, which can reversibly complex proteins via multivalent eosin interactions. PEG-pEosin was able to enhance the intracellular delivery of protein cargos by co-formulation with an endosomal disruptive protein. We anticipate numerous applications of PEG-pEosin due to its ability

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to reversibly package multiple proteins, and shield and release them in active form under mildly acidic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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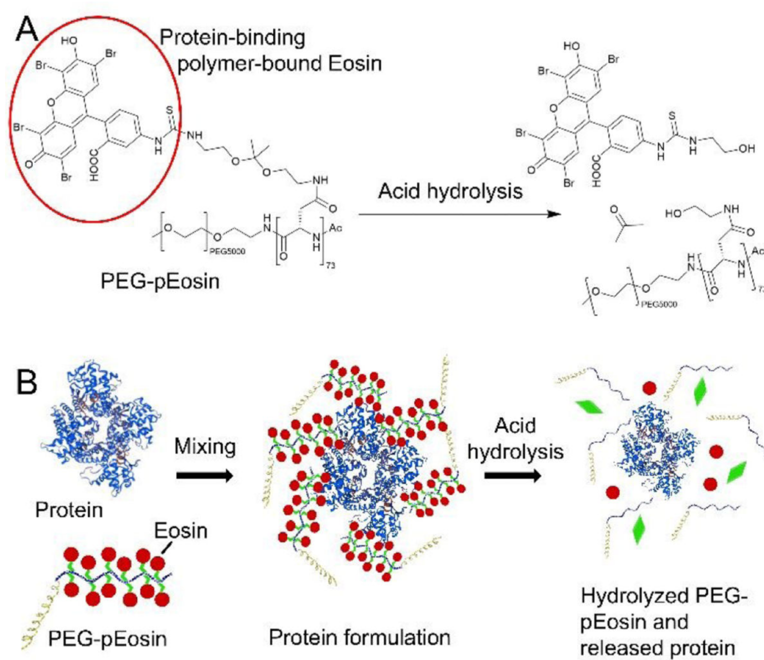

Figure 1: PEG-pEosin is a block-copolymer designed to deliver proteins

(A) Structure of PEG-pEosin and its hydrolysis products. PEG-pEosin is a block copolymer that has a PEG chain and multiple eosin groups. The eosin is attached via acid degradable ketal linkages, and is released from the polymer at acidic pHs. (B) PEG-pEosin binds proteins via multivalent eosin interactions and forms micelles, the proteins are released under acidic conditions, due to hydrolysis of the ketal linkage.

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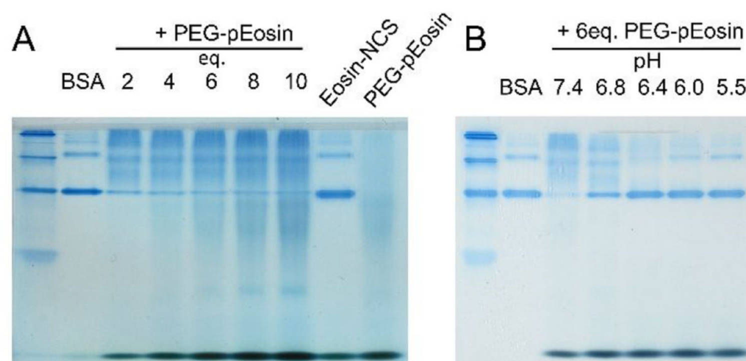

Figure 2: PEG-pEosin binds proteins.

(A) Native PAGE showing protein binding of the PEG-pEosin polymer to bovine serum albumin (BSA) at different molar ratios. PEG-pEosin/BSA molar ratios > 6 result in efficient binding of BSA. (B) PEG-pEosin releases BSA under acidic conditions, due to hydrolysis of the ketal linkage. Incubation of BSA+PEG-pEosin at pHs below 6.8 results in release of BSA.

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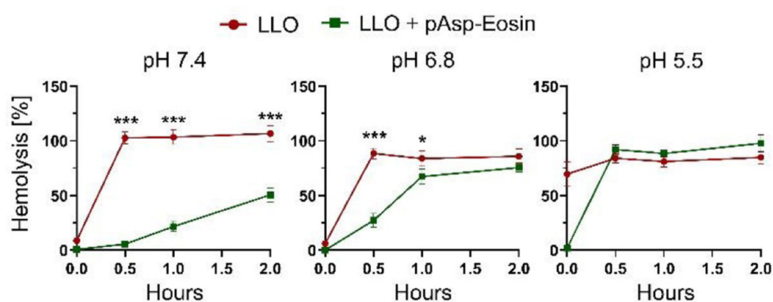

Figure 3: PEG-pEosin reversibly inhibits the activity of LLO.

Hemolysis assay of free and PEG-pEosin-formulated LLO at pH 7.4, 6.8 and 5.5, over a course of 2 hours. PEG-pEosin-formulated LLO has minimal hemolysis as pH 7.4 (30 minutes), but recovers activity at pH 5.5 (30 minutes). Negative control (hemoglobin release from PBS-treated erythrocytes) was set to 0 %. Triton X treatment served as positive control and was set to 100 %. ***: p 0.001, *: p 0.05.

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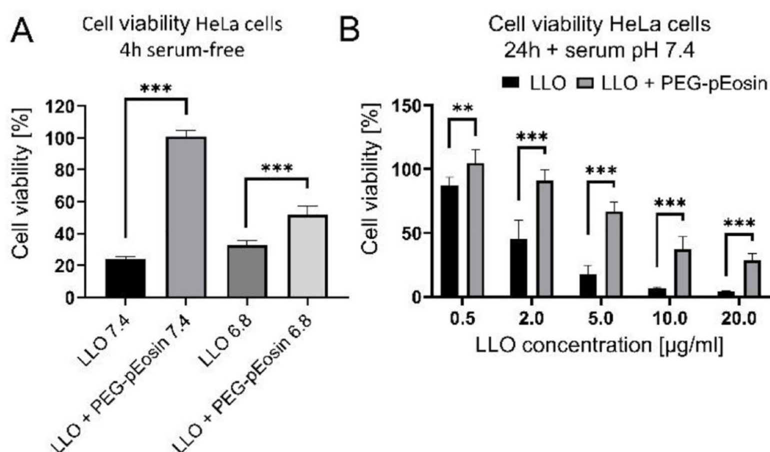

Figure 4: PEG-pEosin formulated LLO is a pH sensitive cytotoxic agent.

Cell viability assay cells treated with free and PEG-pEosin-formulated LLO (A) Cell viability of cells treated with free and PEG-pEosin-formulated LLO at a 2.5 μg/mL concentration, at pH 7.4 and pH 6.8 for 4 hours in serum-free media and (B) Cell viability of cells treated with free and PEG-pEosin-formulated LLO at 24 hours at different concentrations in serumcontaining media. PEG-pEosin-formulated LLO has lower toxicity than LLO at pH 7.4, but at pH 6.8 they have similar cell toxicity. The metabolic activity of cells is shown relative to PBS-treated cells. ***: p 0.001, **: p 0.01.

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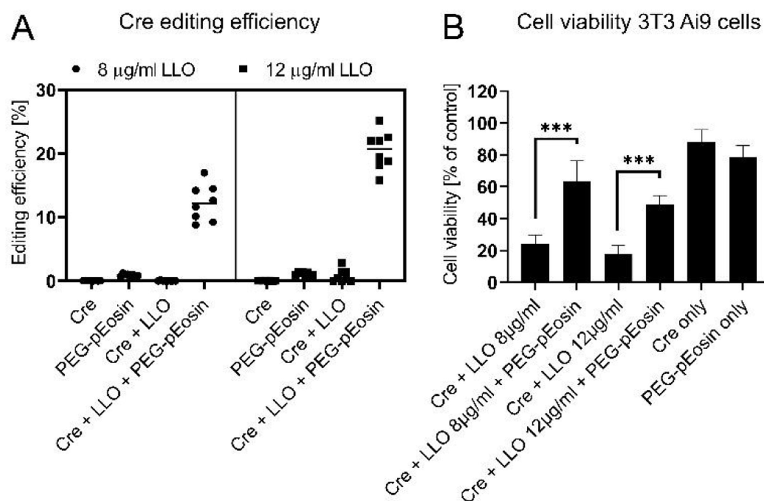

Figure 5: PEG-pEosin-formulated Cre can efficiently edit cells.

(A) Gene editing efficiency of Cre recombinase co-formulated with two different concentrations of LLO and PEG-pEosin. Cre+LLO+PEG-pEosin can edit 3T3 Ai9 cells significantly better than Cre only, PEG-pEosin and Cre + free LLO. Gene editing was determined by flow cytometry and analyzed the TdTomato-positive cells. (B) Cell viability of 3T3 Ai9 cells after incubation with Cre recombinase co-formulations with two different concentrations of LLO and PEG-pEosin, Cre only, PEG-pEosin and Cre + free LLO. ***: p 0.001.

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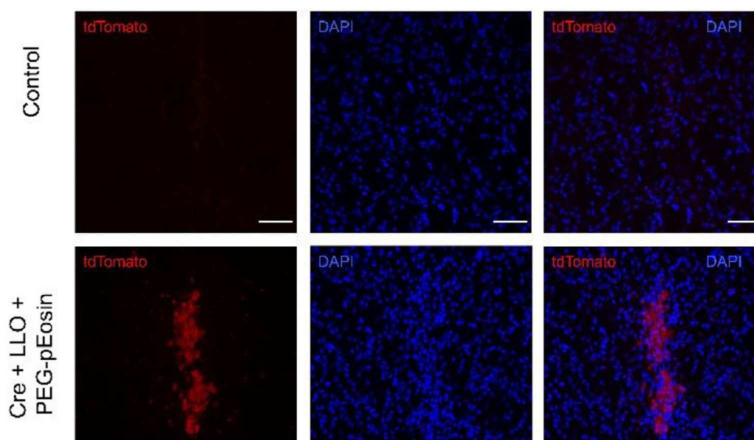

Figure 6: PEG-pEosin formulated Cre can efficiently edit cells.

Cre+LLO+PEG-pEosin (PEG-pEosin: 53.5 μM, LLO: 6.1 μM, Cre: 2.8 μM) was injected into the striatum of Ai9 mice, and analyzed for gene editing, via the expression of TdTomato (red fluorescence), 20 days after the injection. The control was Ai9 mice injected with saline. Ai9 mice treated with Cre+LLO+PEG-pEosin have numerous red cells indicating successful gene editing. Bar represents 50 μm. Blue color = DAPI staining.