PEG–PLA microparticles for encapsulation and delivery of Tat-EGFP to retinal cells

Mehrdad Rafat a,b,1, Carolyne A. Cléroux a,b,1, Wai Gin Fong a, Adam N. Baker a, Brian C. Leonard a, Michael D. O’Connor a,1, Catherine Tsilfidis a,b,1*

a Ottawa Hospital Research Institute, Ottawa Hospital, General Division, 501 Smyth Road, Ottawa, ON, K1H 8L6, Canada
b Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, K1H 8M5, Canada

A R T I C L E   I N F O
Article history:
Received 15 October 2009
Accepted 9 January 2010
Available online 9 February 2010

Keywords:
Biodegradation
Electron microscopy
Gene therapy
Microencapsulation
Polyactic acid
Retina

A B S T R A C T
The efficient and controlled delivery of genes and proteins to retinal cells remains a challenge. In this study, we evaluated polyethylene glycol-polyactic acid (PEG–PLA) microparticles for encapsulation and delivery of a Transactivator of transcription-enhanced green fluorescent protein fusion (Tat-EGFP) to retinal cells. Our main objective was to develop a microparticle system that delivers Tat-EGFP with an initial rapid release (within 24 h) followed by a sustained release. We prepared four different formulations of Tat-EGFP encapsulated PEG–PLA particles to investigate the effects of protein and polymer concentrations on particle morphology and protein release, using scanning electron microscopy (SEM) and fluorometry techniques. The optimum formulation was selected based on higher protein release, and smaller particle size. The optimum formulation was then tested in vitro for cell biocompatibility and protein internalization, and in vivo for cellular toxicity following sub-retinal injections into rat eyes. The results suggest that PEG–PLA microparticles can deliver proteins in cell culture allowing protein internalization in as little as 1 h. In vivo, protein was shown to localize within the photoreceptor layer of the retina, and persist for at least 9 weeks with no observed toxicity.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction
Retinal diseases are a major cause of irreversible blindness. Diseases affecting the retina such as age-related macular degeneration (AMD), diabetic retinopathy, glaucoma, retinal ischemia, and retinal detachment, cause visual loss in millions of people worldwide. Developing an effective therapy is confounded by the fact that some of these diseases (e.g. AMD, diabetic retinopathy, and glaucoma) may be chronic, progressive disorders while others (e.g. retinal ischemia and retinal detachment) are acute insults requiring immediate intervention.

Historically, drugs have been administered to the eye as topical liquid drops. This may be an acceptable mode of delivery for anterior ocular disorders such as corneal or conjunctival diseases. However, diseases of the retina involve cells in the back of the eye. Due to the restricted permeability of the corneal and conjunctival epithelia [1], and the presence of the blood-retina barrier, which limits the efficacy of systemic delivery of therapeutic agents [2], drug delivery to the retina by conventional methods poses a challenge. While it has been demonstrated that intraocular viral delivery can effectively circumvent these barriers and deliver genes to the retina [3–5], viral vectors have been associated with risks of insertional mutagenesis [6,7], immunogenicity, and in some cases death [8]. In addition, viral vectors can persist for years in vivo [9,10], and cannot be deactivated in cases presenting long-term side effects. Non-viral gene delivery (such as plasmid DNA) has also been shown to deliver target genes to the retina [11]. However, this approach is only effective for proteins that can be synthesized at the site of delivery, and would be ineffective for the delivery of other drugs (for example antibodies).

Intraocular drug delivery has been used, but the administered drugs have a limited half-life and are rapidly biodegraded or cleared, limiting their effectiveness. Repeated injections are required, increasing the risk for ocular inflammation and endophthalmitis [12,13]. There is, thus, a vital need for an optimized noninvasive or minimally invasive drug delivery vehicle that can provide sustained intraocular release of therapeutic agents. Moreover, due to the delicate structure of retina, the delivery vehicle must be designed in a nano- or micro-scale size range, to allow both intravitreal and sub-retinal modes of delivery without ocular complications.

Microparticles and nanoparticles have been extensively studied as drug delivery vehicles [14–16]. They offer the advantages of controlled, sustained drug release, sub-cellular size and...
biocompatibility. In the eye, synthetic-based microparticles have been tested for the intravitreal or topical release of therapeutic agents [17,18]. However, as far as we are aware, there has been no report on the use of biodegradable PEG–PLA micro- or nanoparticles for encapsulation and sub-retinal target delivery of proteins or therapeutic agents.

The primary objective of this research was to address the growing need for an effective drug delivery system to prevent retinal degeneration. More specifically, the aim was to develop biodegradable microparticles to encapsulate, transport, and release therapeutic agents to the retina under controlled conditions, in order to prevent or reduce disease progression. Here we report in vitro and in vivo evaluations of PEG–PLA microparticles for encapsulation and delivery of Tat-EGFP to retinal cells. The Tat protein transduction domain is an 11 amino acid domain of the HIV-1 transactivator of transcription protein. It was fused to EGFP to allow cellular internalization of the protein by macropinocytosis [19–21]. Four different formulations of PEG–PLA microparticles encapsulated with Tat-EGFP were fabricated using a 2² factorial experimental design and tested for protein release and particle size. The optimum microparticle formulation with respect to protein release and particle size (MP-3) was then examined in culture using the retina-derived 661 W cell line. 661 W cells are SV40 T-antigen transformed retinal cells that express photoreceptor markers [22] and are thus an appropriate cell line to test in vitro retinal cell delivery. Microparticles were also evaluated in vivo by sub-retinal injection into rat eyes.

2. Materials and methods

2.1. Materials

PEG–PLA di-block polymer (PEG(1000)-b-PLA(5000)) was purchased from Polysciences, Inc. (Warrington, PA). Numbers in parentheses refer to the molecular weight (MW) of the segment. Polyvinyl alcohol (PVA), MW ~ 78000, 99.7 mol% hydrolyzed, was obtained from Polysciences, Inc. Dichloromethane was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Synthesis of Tat-EGFP

The pET28a Tat-EGFP vector was constructed by PCR amplification using a 5′ primer containing a BamHI restriction site, the 11 amino acid Tat protein transduction domain and the first 19 nucleotides of the EGFP-cDNA. The vector adds a His tag in frame to the 5′ end of Tat-EGFP. BL21 E. coli containing the pET28a Tat-EGFP plasmid was grown overnight in 1 L of Terrific broth at 22 °C until use. The bacterial pellet was lysed in 50 ml of solubilization buffer (50 mM Tris–Cl (pH8.0), 150 mM NaCl, 8 M urea and 25 mM imidazole; Sigma) and sonicated with 150 mM imidazole and dialyzed three times in 4 L PBS. Protein was concentrated in solubilization buffer. Tat-EGFP was eluted in solubilization buffer supplemented with 4 M. Rafat et al. / Biomaterials 31 (2010) 3414–3421

Table 1

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Factors</th>
<th>Actual values for factors*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X1</td>
<td>X2</td>
</tr>
<tr>
<td>MP-1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MP-2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>MP-3</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>MP-4</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Factors and their levels were chosen based on bench-top experiments as well as the reports in the literature.

2.3. Preparation of PEG–PLA microparticles

PEG–PLA microparticles loaded with Tat-EGFP protein at different particle and protein concentrations were prepared by a Water/Oil/Water (W/O/W) emulsion or double emulsion solvent evaporation method [23,24]. Microparticles were used to encapsulate Tat-EGFP in order to serve as a stabilizer/carrier and to control release of the protein. Four different formulations of PEG–PLA microparticles were developed (MP-1 to MP-4) (see Table 1). For example, for the MP-3 formulation, 2 ml at (0.6% w/v) of Tat-EGFP in PBS was dispersed in 6 ml of a 2% w/v solution of PEG–PLA using a sonicator. The resulting primary emulsion was poured into 40 ml of 1% aqueous PVA solution, homogenized to form a secondary emulsion, and stirred for 3 h for stabilization of particles and evaporation of the organic solvent. The solidified microparticles were collected by centrifugation, washed three times with PBS, frozen at −20 °C overnight, lyophilized for 7 h, and stored at 4 °C until use.

2.4. Factorial experimental design for preparation of PEG–PLA microparticles

A 2² full factorial design was planned to investigate the impact of two key factors including Tat-EGFP concentration and PEG–PLA polymer concentration on morphology, and protein release kinetics. As listed in Table 1, the key factors and their levels were determined based on the information available in the literature and a series of bench-top exploratory experiments performed prior to the factorial design. The number of possible treatments (compositions) was determined to be four (2²).

2.5. Scanning electron microscopy (SEM)

The morphology of the freeze-dried PEG–PLA microparticle formulations was investigated using a scanning electron microscope (SEM, Model S-2250N, Hitachi, Japan). The particles were mounted on metal holders using conductive double-sided tape, and sputter coated with a gold layer for 60 s at 0.1 bar vacuum pressure (Cressington Sputter Coater, 108) prior to SEM examination.

2.6. In vitro release of Tat-EGFP from PEG–PLA microparticles

Microparticles were dispersed in DMEM high glucose media (HyClone, Thermo Scientific, Waltham, MA), supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 10,000 units/ml Penicillin and 10 mg/ml Streptomycin (Gibco, Invitrogen Life Technologies, Carlsbad, CA), 2 mM l-glutamine (Gibco) and 1 mM sodium pyruvate (Sigma) (supplemented media will hereafter be referred to as “culture media”). Dispersed particles were aliquoted into 2 ml microcentrifuge tubes in triplicate, and incubated at 37 °C with agitation to allow protein release over time. Samples were obtained by centrifuging tubes at 15 K rpm in a microcentrifuge for 10 min, collecting the supernatant, and replacing it with fresh culture media. A 100 μl aliquot of each sample was read for Tat-EGFP fluorescence at 509 nm following excitation at 480 nm using a BioTek Synergy HT plate reader. Tubes containing media free of microparticles were also subject to the same manipulations and read as negative controls. Samples containing a 0.5 μg/ml solution of Tat-EGFP in culture media were used as a positive control. Samples were taken at 3 h, 6 h, 24 h, and every 3 days afterward for up to 31 days. Tat-EGFP was quantified by referencing fluorescence readings to a linear standard curve generated from known Tat-EGFP concentrations. To confirm results, samples were analyzed for Tat-EGFP content by an enzyme-linked immunosorbent assay (ELISA). The Abcam® sandwich ELISA protocol was followed. Immulon® 2HB 96-well microtiter plates (Thermo Scientific) were used. A polyclonal goat anti-GFP antibody (Abcam, Cambridge, MA) at a concentration of 1 μg/ml was used as a capture antibody. The detection antibody used was a rabbit anti-GFP IgG fraction (Invitrogen) diluted 1/1000, and the secondary antibody was an alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at 1/5000. A 1% BSA solution was used as a blocking agent. Detections were performed by adding 100 μl p-nitrophenyl phosphate (pNPP; Calbiochem, EMD Biosciences, San Diego, CA) dissolved in the appropriate buffer to each well, and incubating for 15–60 min. The reaction was stopped by adding 100 μl 1 N NaOH, and absorbance was read at 405 nm.

2.7. In vitro release of Tat-EGFP from PEG–PLA microparticles in cellular culture

Photoreceptor-derived 661 W cells were cultured in 24-well plates on microscope cover glass, and incubated at 37 °C in cell culture media. The following day, media was replaced with a suspension of PEG–PLA microparticles (MP-3 formulation) at 500 μg/ml in culture media. Fresh media devoid of microparticles was used as a negative control, and a 50 μg/ml suspension of Tat-EGFP protein in culture media as a positive control. Cellular uptake of released Tat-EGFP was assessed by immunocytochemistry. After 1 h, 24 h, 48 h, and 96 h of incubation with microparticles, cells were washed with PBS and fixed in 4% PFA, followed by four 5-min washes in PBS. Cells were incubated overnight at 4 °C with primary antibody (1/1000 rabbit anti-GFP IgG fraction (Invitrogen) with 100 μg/ml BSA, 1% goat serum and 0.2% Triton X-100). Cells were then washed in PBS and incubated with secondary antibody (Alexafluor 488 goat anti-rabbit IgG, Molecular Probes, Invitrogen) for 1 h at room temperature, with agitation. Cells were washed four more times in PBS, and counterstained with 1/2000 DAPI during the second wash. Cover glasses were then mounted on slides in antifade (50:50 PBS:Glycerol with 1% N-propyl Gallate). Images were taken by fluorescence microscopy (Zeiss Axioskop2 equipped with an Axioscan HRC digital camera, Carl Zeiss, Germany).
2.8. In vivo injections of PEG–PLA microparticles

Adult female Long-Evans rats were purchased from Charles River Laboratories (Wilmington, MA). Animals were maintained under standard laboratory conditions and all procedures conformed to both the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the University of Ottawa Animal Care and Veterinary Service. Tat-EGFP-loaded PEG–PLA microparticles (MP-3 formulation) were redispersed in sterile DPBS (Hyclone, Thermo Scientific) at a concentration of 10 mg/ml using sonication for 180 s at a power setting of 10 W. Injections were carried out in the left eye of 18 rats. Six animals were injected with Tat-EGFP encapsulated PEG–PLA microparticles (MP-3), six with empty microparticles of the same composition, and six with an adenovirus–associated virus (AVV) expressing GFP driven by the chicken beta actin (CBA) promoter at a titer of 5.6 x 10^13 particles/ml. The right eyes served as un.injected controls. Injections were performed under isoflurane anesthesia. Two microliters of the microparticle or viral suspension was delivered into the sub-retinal space, according to our previous methods [25]. One week post-injection, treated eyes were observed for signs of retinal detachment, cataracts or other ocular complications. Three weeks and nine weeks post-injection, fundus images were taken of a subset of injected eyes, using the Retcam II wide-field digital imaging system (Clarity Medical Systems, Pleasanton, CA). Electroretinography (ERG) was performed at nine weeks post-injection to test for altered retinal function. ERGs were generated using the ESPION system (Diagnosys, Inc., Hampshire, England) according to our previous protocols [26].

2.9. Sampling, tissue processing and immunohistochemistry

Animals were sacrificed and the eyes sampled at nine weeks post-injection. Rats were injected intraperitoneally with sodium pentobarbital and trans-cardially perfused with 4% PFA as reported in [27]. Cryosections (10 μm) were prepared using a Shandon cryostat (Thermo Scientific). Sections were air dried for 2 h, and stored at −20 °C with desiccant. Immunohistochemistry was performed on cryosections using rabbit anti-GFP IgG (Invitrogen) as a primary antibody, and Alexafluor 488 goat anti-rabbit IgG (Molecular Probes, Invitrogen) as a secondary antibody. Slides were post-fixed in 4% PFA, and blocked in 1% BSA in TBS with 5% goat serum. They were incubated in a humidified chamber at 4 °C overnight with the primary antibody (1/200) in block solution with 0.2% TritonX-100, and for 60 min at room temperature with the secondary antibody (1/200). All washes were done in TBS. Slides were counterstained with DAPI in the final washes, and mounted with coverslips with antifade.

3. Results and discussion

3.1. SEM characterization

SEM micrographs of the four PEG–PLA formulations suggest that PEG–PLA polymer and Tat-EGFP protein concentrations had significant impacts on morphology of particles (Fig. 1). For example, by increasing Tat-EGFP concentration from 0.3 to 0.6%, particle size decreased (compare MP-3 to MP-1, and MP-4 vs MP-2). The charge-dipole interactions between negatively-charged PEG–PLA molecules and positively-charged Tat-EGFP may have contributed to morphology changes. At a fixed polymer concentration, a higher protein concentration may result in a higher population of oppositely charged molecules that may increase coagulation and particle formation rate resulting in smaller particles (e.g., MP-3 vs. MP-1 and MP-4 vs. MP-2) whereas like-charged molecules may inhibit or decrease the coagulation and particle formation process [28,29].

As for the effect of polymer concentration on particle morphology, we observed that an increase in PEG–PLA concentration from 2% to 4% disrupted particle formation resulting in irregularly shaped particles (compare MP-2 to MP-1, and MP-4 vs MP-3; Fig. 1). This phenomenon may be caused by the polar interactions and population imbalance between oppositely charged molecules.

3.2. Tat-EGFP release from microparticles

Over a period of 31 days, the cumulative release of the four PEG–PLA formulations demonstrated a biphasic release profile that included an initial burst in the first 24 h of incubation followed by a slow but sustained release phase (Fig. 2). This biphasic release profile is typical of PEG–PLA microparticles, and has been reported in the literature [30–33]. The initial burst phase is associated with the release of protein at the particle surface, while the sustained phase represents the slow diffusion of protein from the particle core and the slow release of protein as the particles degrade. The MP-3 formulation had the highest cumulative release that was mostly attributed to its high initial burst, followed by MP-4, MP-2, and MP-1. By comparing the release profiles of MP-3 and MP-4 to those of MP-1 and MP-2, respectively, it can be seen that the cumulative protein released is directly proportional to the concentration of protein initially used for encapsulation. No consistent relationship was observed between the cumulative protein release and the polymer concentration. For example, at a low protein concentration of 0.3%, the polymer concentration had an increasing impact on the cumulative release (MP-2 vs. MP-1)

---

Fig. 1. Scanning electron microscopy (SEM) images of microparticle formulations MP-1 (A), MP-2 (B), MP-3 (C) and MP-4 (D) depicting the effects of polymer and protein concentrations on particle morphology. The MP-3 formulation (0.6% protein, 2% polymer) has the smallest microparticles.
while at a high protein concentration of 0.6%, the polymer concentration had a decreasing impact on the cumulative release profile (MP-4 vs. MP-3).

3.3. Selection of the optimum microparticle formulation

The optimum formulation was selected based on the smallest particle size and the highest cumulative protein release compared to the other three formulations. Particle size is an important factor for sub-retinal delivery. The delicate nature of the retina and the need for intimate contact between the outer retina (the photoreceptors) and the underlying retinal pigment epithelium prevents the use of large particles that would cause a retinal detachment and lead to photoreceptor cell death. The second important factor in selecting the optimum formulation is the protein release profile. Given the acute and/or chronic nature of retinal diseases, it is important to develop a release mechanism through which a large amount of the protein or gene can be delivered initially followed by a controlled slow release. In the case of acute insults such as retinal detachment and retinal ischemia, rapid delivery and upregulation of the drug is crucial given the rapid death of the retinal neurons. In the case of chronic diseases such as AMD and diabetic retinopathy, a sustained delivery is more important.

The MP-3 formulation demonstrated a better release profile and smaller sized particles in comparison with the other formulations. The MP-3 optimum formulation was further investigated for other properties, including dispersion in media, cell biocompatibility, protein internalization, and in vivo release and toxicity after sub-retinal injections into rat eyes.

3.4. Effect of sonication parameters on particle dispersion and Tat-EGFP release

The effects of sonication duration and sonicator output power were investigated on particle dispersion and protein release (Fig. 3). Lyophilized microparticles (MP-3 formulation) were dispersed in PBS at a concentration of 6 mg/mL as per the method described in Section 2.6. The extent of dispersion and release of the microparticles were investigated by fluorescence microscopy imaging (excitation: 488, emission: 509) and protein release assays, respectively. Sample A of microparticles was dispersed in PBS using a sonicator for 25 s at a power output of 3 W (Fig. 3A), while sample B was dispersed in PBS using a sonicator for 180 s at a power output of 10 W (Fig. 3B).

![Fig. 3. Effects of sonication time and power on microparticle dispersion and Tat-EGFP release for PEG–PLA MP-3 formulation: (A) Fluorescence image of microparticles in PBS (sonication time = 25 s, power = 3 W), (B) Fluorescence image of microparticles in PBS (sonication time = 180 s, power = 10 W), (C) Effects of sonication time and power on Tat-EGFP release.](image-url)
Fig. 4. Cellular uptake of Tat-EGFP released from PEG–PLA microparticles in mouse photoreceptor-derived 661 W cells. 500 μg of microparticles suspended in culture media were added to each cell culture and incubated at 37 °C. Fluorescence microscopy and Differential Interference Contrast (DIC) images were taken after 1 h (A, B), 24 h (C, D), 48 h (E, F) and 96 h (G, H). Control cells were cultured in the presence of 50 μg/mL of Tat-EGFP for 1 h (I, J) or without protein or microparticles (K, L).
Fluorescence micrographs (Fig. 3A and B) suggest the presence of Tat-EGFP in the microparticles (under UV excitation, Tat-EGFP encapsulated particles emitted a green fluorescence corresponding to Tat-EGFP’s emission peak at 509 nm). At a high sonication power and time (Fig. 3B), particles were better dispersed, and large clusters of particles (as shown in Fig. 3A) were not present.

The protein release profile was increased by increasing sonication time and power (Fig. 3C). This phenomenon is likely attributed to better particle dispersion and an increase in effective surface area of particles, which facilitates particle degradation and protein diffusion out of the particles.

3.5. Cellular internalization of Tat-EGFP released from microparticles

661 W cells were incubated in the presence of Tat-EGFP encapsulated PEG-PLA microparticles (dispersed in the culture media). Samples were tested for cellular uptake of released Tat-EGFP after 1 h, 24 h, 48 h, and 96 h of incubation. Fluorescence microscopy and phase contrast images are shown in Fig. 4. Incubation of cells with 50 µg/mL Tat-EGFP protein in media confirmed that cellular uptake of Tat peptides occurs within 1 h (Fig. 4I). In microparticle-treated samples (MP), a fainter, more diffuse signal was observed in the cell cytoplasm after 1 h of incubation, indicative of rapid Tat-EGFP uptake by 661 W cells (Fig. 4A). These results concur with early findings that Tat peptides can enter cells within 10 min [37]. A study by Caron et al. (2001) reported internalization of a Tat-EGFP fusion protein by cultured myoblasts reaching maximum intracellular levels within 30 min of incubation [38]. The weak cytoplasmic signal (Fig. 4A and C) may be attributed to the initial burst of protein released from the microparticles upon resuspension, as seen in the in vitro release experiments (Fig. 2). While at early time points the microparticles did not appear to specifically co-localize with cells, by 24 h, however, a strong Tat-EGFP signal was observed on the cells, suggesting the association of microparticles with the cell surface (Fig. 4C). After 48 and 96 h of incubation, stronger Tat-EGFP signals were observed in the cell cytoplasm compared to 1 h and 24 h time points, suggesting the potential internalization of microparticles and/or the released Tat-EGFP (Fig. 4E and G). Complementary assays performed on HEK293 (human embryonic kidney) cells suggest no toxicity of microparticles (see Supplemental Fig. S1)

3.6. Tat-EGFP release and internalization in vivo

Three groups of six rats each received sub-retinal injections of MP-3 microparticles loaded with Tat-EGFP, empty microparticles (no
Tat-EGFP, or AAV-CBA-GFP virus in their left eye. Ophthalmoscopic observations one week post-injection revealed what appeared to be membranes in the vitreous, rising from the retinal puncture site in most animals injected with microparticles. Few eyes showed retinal detachment, and all the lenses appeared clear. Fundus images were taken at 3 and 9 weeks post-injection on a subset of the injected animals (Fig. 5). At both time points, no lens opacity was observed. Particulate matter could be seen in the sub-retinal space in the microparticle-injected retinas (Fig. 5A, B, D and E), and thin ‘needle tracks’ rising from the puncture site were visible in most microparticle-injected eyes (Fig. 5B and E), although they appeared much smaller by 9 weeks. These “needle tracks” are also sometimes seen in the clinic following sub-retinal drug delivery in retinal disease patients. The fundus images showed the persistence of the particles in the sub-retinal space 9 weeks post-injection, suggesting that they could be useful for the delivery of compounds in the eye for durations of at least 2 months following a single injection.

Electroretinograms (ERGs) were taken at various light intensities to test retinal function. A-waves (indicative of photoreceptor function) and b-waves (indicative of the health of interneurons that synapse with photoreceptors) showed no significant differences between PEG–PLA-treated eyes and control eyes (Fig. 6). The normal retinal function of microparticle-injected retinas supports the biocompatibility and absence of toxicity of PEG–PLA microparticles.

Immunostaining of retinal sections sampled at 9 weeks post-injection revealed the presence of Tat-EGFP protein in the photoreceptor outer nuclear layer (ONL) (Fig. 7). Aggregates of Tat-EGFP protein within this layer also suggested that most of the protein was still contained within microparticles, some of which had infiltrated the retinal outer nuclear layer (Fig. 7A and B). A similar, but more intense localization was seen following injections of an AAV-GFP viral vector (Fig. 7C and D). These results support the efficacy of PEG–PLA microparticles for the delivery of proteins targeting photoreceptor cells. The concentrations of the protein were somewhat low in comparison to AAV expression levels, but this may not be a concern in treatments where nanogram quantities of protein are sufficient for neuroprotection.

Fig. 7. Immunohistochemistry and DIC images of injected retinas. DAPI counterstain (blue) identifies the neuronal nuclei. Eyes were subretinally injected with Tat-EGFP-loaded PEG–PLA microparticles (MF-3 formulation), and sampled at 9 weeks post-injection (A, B). Green fluorescence shows the localization of Tat-EGFP within the nuclei of the photoreceptor layers (arrow heads in A and inset). An eye injected with an AAV-CBA-GFP viral construct is shown for comparison (C, D). The negative control is an un.injected right eye (E, F).
4. Conclusions

The current study shows that PEG–PLA microparticles designed with the MP-3 formulation (1 kDa PEG, 0.6% protein, and 2% polymer concentrations) can effectively deliver proteins to the outer segment of the retina, without any apparent cytotoxic effects. PEG–PLA microparticles show great potential for sustained ocular drug delivery because of their high stability in vitro and in vivo, and their release profile (exhibiting a burst of release in the initial stages followed by slow, sustained release over several weeks). They allow for targeted delivery to cells at the back of the retina (i.e., photoreceptor cells), which could prove effective in the treatment of blinding diseases caused by photoreceptor cell loss.

Acknowledgements

The authors wish to thank Dr. Joseph Karov (Health Canada) for his help and advice on SEM work, Dr. William Hauswirth (University of Florida) for the AAV virus and Dr. Frank Li (University of Ottawa) for advice on microparticles chemistry. We greatly appreciate Health Canada's co-operation in allowing us access and use of their SEM system. This work was funded by a Canadian Institutes of Health Research grant to CT. CC was supported by an Ontario Graduate Scholarship.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2010.01.031.

Appendix

Figures with essential colour discrimination. Figs. 3–7 in this article may be difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2010.01.031.

References