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Original Article

In vitro and *in vivo* assessment of delivery of hydrophobic molecules and plasmid DNAs with PEO–PPO–PEO polymeric micelles on cornea

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ABSTRACT

The stability and bio-distribution of genes or drug complexes with poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO–PPO–PEO, Pluronic F-68) polymeric micelles (PM) are essential for an effective nanosized PM delivery system. We used Förster resonance energy transfer (FRET) pairs with PM and measured the FRET ratio to assess the stability of PM *in vitro* and *in vivo* on the cornea. The FRET ratio reached a plateau at 0.8 with 3% PM. Differential scanning calorimetry measurement confirmed the complex formation of FRET pairs with PM. Confocal imaging with the fluorophores fluorescein isothiocyanate isomer I (FITC) and rhodamine B base (RhB) also showed the occurrence of FRET pairs *in vitro*. The fluorophores were mixed with 3% PM solution or the FITC-labeled PEO–PPO–PEO polymers (FITC-P) were mixed with RhB-labeled plasmids (RhB–DNA). In addition, the *in vitro* corneal permeation of FRET pair complexes with PM reached a 0.8 FRET ratio. One hour after eye drop administration, FRET pairs colocalized in the cytoplasm, and surrounded and entered the nuclei of cells in the cornea, and the polymers were located in the corneal epithelial layers, as detected through anti-PEG immunohistochemistry. Furthermore, fluorescence colocalization in the cytoplasm and cell nucleus of the corneal epithelium was confirmed in tissues where RhB or RhB–DNA complexed with FITC-P was found to accumulate. We demonstrate that at a concentration of 3%, PM can encapsulate FRET pairs or RhB–DNA and retain their integrity within the cornea 1 h after administration, suggesting the feasibility and stability of PEO–PPO–PEO polymers as a vehicle for drug delivery.

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1. Introduction

Biocompatible poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide), known as PEO–PPO–PEO triblock copolymers, have drawn increasing attention as a pharmaceutical application because of the hydrophilic PEO ends that provide a steric barrier against opsonization [1,2]. In addition, the self-assembly of the hydrophobic core of PEO–PPO–PEO block copolymers provides a microenvironment for water-insoluble molecules and is believed to facilitate solubility in polymeric micelles (PM) [1]. Thus, PEO–PPO–PEO PM have been used as carriers for the IV injection of the anti-inflammatory agent (methylprednisolone) [3] and the antineoplastic agent (doxorubicin) [4], as well as oral non-viral gene delivery [5], intramuscular sustained release formulations [6], and transdermal patch or inhalation applications [7,8].

Stability is a predominant factor that affects the efficiency of PM carrier systems. In contrast to relatively stable nanocarriers with solid-like cores, it remains unclear whether PM dissociates to free polymer chains and attains dynamic equilibrium after extreme dilution. Because the concentration of PM carriers in a biological system is likely to decline to a level below the critical micelle concentration (CMC), the minimum concentration at which amphiphilic polymers assemble to form PM, real-time monitoring of the micelle status and drug retention within the PM is necessary for successful delivery [9]. Förster resonance energy transfer (FRET) between two fluorescent probes is used to determine when two molecules are close to one another on a molecular scale (<80 Å), as well as whether both molecules are moving relative to each other [10]. The FRET ratio is generally used to determine whether two fluorophores are within a certain distance of each other. Thus, FRET has been extensively used as a spectroscopic ruler for nanosized particles to determine fluorophore proximity and the release of hydrophobic molecules from carriers [11].

Visual impairment caused by trauma, macular degeneration, diabetic retinopathy, or glaucoma, is the leading cause of blindness worldwide. According to the World Health Organization, an approximately 39 million people out of 285 million cases of visual impairment in 2010 were cases of blindness [12]. Delivery of anti-inflammatory [13], antiglaucoma drugs, or gene therapy to the eyes provides additional options for ophthalmic treatments. Topical instillation of ophthalmic drops is the most common method of administering drugs to treat ocular disease. PEO–PPO–PEO has been approved by the United States Food and Drug Administration for use in ophthalmic pharmaceuticals [14]. However, drug delivery to the eyes has remained a challenging task, due to the difficulties associated with absorption of topical drugs in the tightly structured corneal epithelium and the anteriorly directed aqueous humor bulk flow. The other major barrier to the development of drugs composed of small molecules or genes is the instability of the process. Such instability can lead to immunogenicity and loss of activity, such as genes being further destabilized by nucleases [15].

Although PM has been used as topical ocular vehicles to facilitate delivery of therapeutic genes to the cornea [16–18], it is critical to illustrate the delivery pathway of PM to determine whether the integrity and stability of the PM complex and its cargo remain intact [19,20]. Obstacles that PM could encounter after penetrating the cornea includes tear flow which has a high turn-over rate, and the stratified multi-layered corneal epithelium which has high resistance barrier to foreign substances [21]. Therefore, we applied a fluorogenic-based approach with two hydrophobic fluorophore FRET dyes: fluorescein isothiocyanate isomer I (FITC) and rhodamine B base (RhB). We took advantage of the transparent characteristic of the cornea to investigate the stability and biodistribution of PM and its cargo, by using free hydrophobic fluorophore molecules, fluorophore-labeled DNA (RhB–DNA), or PEO–PPO–PEO block copolymers.

2. Materials and methods

2.1. Materials

PEO–PPO–PEO copolymers (PEO/PPO/PEO = 76/30/76, Pluronic F-68), with an average molecular mass of 8400 Da, were purchased from BASF (Ludwigshafen, Germany). FITC and RhB were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and used as received. Chemical conjugation and purification of the PEO–PPO–PEO with FITC (FITC-P) moiety at the chain end were performed by following the procedures from our previous studies [22].

2.2. Animals

Six- to eight-week-old male nude mice (BALB/c-nu), as the selection of the animal model of the previous gene delivery experiment [17], were obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The mice were maintained under specific pathogen-free conditions and housed under standard conditions with a 12-h light–dark cycle. The protocols on the use of animals were approved by the Laboratory Animal Research Committee of Taipei Medical University.

2.3. Plasmid DNA labeling

A plasmid, pCMV-bcl-xL-eGFP (5.7 kb), was constructed according to previous protocols [18] and amplified in the *Escherichia coli* DH5α strain. The plasmid was purified using equilibrium centrifugation with a CsCl–EtBr gradient. RhB was covalently attached to 100 μg of pCMV–bcl-xL–eGFP DNA using a *Label* IT Nucleic Acid Labeling Kit (Mirus Corp., Madison, WI, USA). *Label* IT Reagent (100 μl) in the buffers was mixed with DNA, and the mixture was incubated at 37 °C for 2 h in the dark. Unincorporated dye was removed, and labeled DNA was purified using ethanol precipitation. Concentrations of DNA were measured through UV absorption. Labeling efficiencies were estimated to be one label for every 60 base pairs, by using a spectrophotometric assay and following a protocol provided by the manufacturer.

2.4. Preparation of PEO–PPO–PEO PM

All concentrations of polymeric solution were prepared on a weight percentage basis according to previous protocols

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