International Journal of Pharmaceutics 365 (2009) 34–43

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

# Biodegradable and biocompatible thermosensitive polymer based injectable implant for controlled release of protein

Yu Tang, Jagdish Singh*

*Department of Pharmaceutics, North Dakota State University, Fargo, ND 58105, USA*

ARTICLE INFO

*Article history:*
Received 30 May 2008
Received in revised form 30 July 2008
Accepted 13 August 2008
Available online 22 August 2008

*Keywords:*
Thermosensitive polymer
Biocompatibility
Controlled release
Lysozyme
Protein stability
Protein delivery

ABSTRACT

This study was aimed at developing a suitable controlled release system for proteins by modifying the structure of thermosensitive copolymer monomethoxy poly(ethylene-glycol)-*co*-poly(D,L-lactide-*co*-glycolide)-*co*-monomethoxy poly(ethylene-glycol) (mPEG–PLGA–mPEG). Eleven mPEG–PLGA–mPEG copolymers were synthesized and characterized by $^1$H NMR and gel permeation chromatography (GPC). Thermosensitivity of the copolymers was tested using the tube inversion method. Four of the eleven synthesized copolymers were dissolved in water as injectable solutions at room temperature which turned into gels abruptly at body temperature (37 °C), indicating the potential use as *in vivo* drug delivery system. Lysozyme was used as a model protein to study *in vitro* release characteristics of the copolymer based delivery system. The copolymer based formulations released lysozyme (quantified by micro-BCA protein assay) over 10–30 days, depending on copolymer structure. The released lysozyme was confirmed to conserve its structural stability by differential scanning calorimetry (DSC) and circular dichroism (CD), and biological activity by specific enzyme activity assay. Furthermore, the copolymer based formulations showed excellent biocompatibility as tested by MTT assay and *in vivo* histological evaluation. Therefore, the copolymers controlled the *in vitro* release of lysozyme while conserving protein stability and biological activity, indicating that it is an appropriate delivery system for long term controlled release of proteins.



## 1. Introduction

There are several therapeutically important proteins that have been discovered in the past two decades since the completion of human genome sequencing and functional studies (Venter, 2001); meanwhile, the tremendous advancements in biotechnology made the large-scale production of these proteins a reality (Lee, 2002). Unfortunately, the clinical application of many of these therapeutic proteins is limited by the lack of suitable delivery systems. So far, delivery by subcutaneous injection is the most commonly used method of administration of protein. In order to maintain the therapeutic effect, frequent injection is required, due to the short half-life of proteins *in vivo* (Pitt, 1990), but low patient compliance makes this administration method undesirable. Thus, a suitable delivery system of protein is warranted in order to deliver protein continuously in its active form over a longer period after a single injection.

Recently, thermosensitive polymer-based injectable *in situ* gel forming drug delivery systems have attracted great research interest because of their advantages, such as ease of manufacturing, avoidance of organic solvents, convenient application, and sustained release of incorporated drug (Hoffman, 1987; Stile et al., 1999). Poly(*N*-isopropylacrylamide) (poly-NIPAAM) is the prototype of thermosensitive polymers. However, due to its toxicity, poly-NIPAAM is not used for drug delivery (Bae et al., 1987; Schild, 1992). The first thermosensitive polymer approved by the FDA was triblock poly(ethylene oxide)-*co*-poly(propylene oxide)-*co*-poly(ethylene oxide) (PEO–PPO–PEO) copolymer, also called "Pluronics®" or "Poloxamer" (Merril and Pekala, 1987; BASF, 1993). Nonetheless, except for its use as surfactant and thickening agent in pharmaceutical products, PEO–PPO–PEO failed to meet expectations for pharmaceutical implants, due to non-biodegradability and occurrence of side effects (Wang and Johnston, 1991; Muller et al., 1997; Wasan et al., 2003). In 1997, MacroMed Inc. developed a type of biodegradable thermosensitive triblock copolymer by replacing the hydrophobic non-degradable PPO block of poloxamer with a biodegradable poly(D,L-lactide) (PLA) block. The new PEO–PLA–PEO ($M_w$ 5000–2040–5000 Da) triblock copolymer was found to control the release of dextran, a high molecular weight hydrophilic molecule, for 12 days (Jeong et al., 1997). Although the PEO–PLA–PEO/water system exhibits sol–gel transition at body temperature (37 °C), this system can only be loaded with

* Corresponding author. Tel.: +1 701 231 7943; fax: +1 701 231 8333.
*E-mail address:* jagdish.singh@ndsu.edu (J. Singh).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.08.018

therapeutic agents at an elevated temperature of 45 °C at which it exists as a Newtonian fluid. This physicochemical property limits its application in protein delivery since most proteins get denatured at high temperatures.

Later, MacroMed Inc. discovered that the thermosensitive sol–gel transition of the copolymer/water system is directly related to the block composition and arrangement of the copolymer (Jeong et al., 1999a,b). After replacing the PLA block with PLGA block, modifying the block length of PEO, and rearranging the block sequence, MacroMed reported that both mPEG–PLGA–mPEG and PLGA–PEG–PLGA (Regel®) are thermosensitive liquid drug carrier systems which can be loaded with therapeutic agents at a temperature lower than 30 °C with a sol–gel transition properties at 37 °C. PLGA–PEG–PLGA (Regel®) has been studied extensively, due to the ease of one-step synthesis. Unfortunately, Regel® system is usually required to be prepared at the temperature lower than room temperature for injectability and was reported to control the release of loaded protein for only ~7 days, further increasing the length of hydrophobic PLGA block caused protein aggregation in the formulations (Zentner et al., 2001; Chen et al., 2005a).

Due to the different gelation mechanism from Regel®, mPEG–PLGA–mPEG/water system was claimed to be able to load with therapeutic agents at room temperature with abrupt sol–gel transition at ~30 °C. Such a special sol–gel transition of the mPEG–PLGA–mPEG/water system not only avoids the high temperature denaturation of loaded protein but also provides convenience in administration because of the system's suitable injectability at room temperature. mPEG–PLGA–mPEG ($M_w$ 550–2810–550 Da) was found to continuously release a hydrophilic drug, ketoprofen, for 3 days and another larger mPEG–PLGA–mPEG ($M_w$ 12,798 by GPC) was reported to release loaded pDNA for ~14 days (Jeong et al., 2000; Li et al., 2003). So far, no further investigation has been published for the application of mPEG–PLGA–mPEG in the controlled release of proteins. According to the reported literature, the block length of biodegradable triblock copolymers has significant effects on the initial burst and the release duration of incorporated therapeutic agents, for two reasons: (1) proper ratio of the length of hydrophilic mPEG block and hydrophobic PLGA block induces less push-out effect by increasing gel's stability, and (2) larger PLGA block results in a controlled release for longer duration of loaded therapeutic agents by slower degradation (Jeong et al., 1999a,b; Chen et al., 2005a; Chen and Singh, 2005b). Thus, extension of the length of PLGA block while conserving the thermosensitivity of the copolymer and stability of formed gel is necessary to produce a suitable delivery system for controlled release of macromolecules over a long period.

The purpose of the present study was to further modify the block length of mPEG–PLGA–mPEG copolymer for extending the release of incorporated protein and to investigate the effects of the copolymers on protein stability. Eleven mPEG–PLGA–mPEG triblock copolymers were synthesized having serially increased length of both hydrophilic mPEG and hydrophobic PLGA block, in order to find a copolymer consisting of the longest hydrophobic PLGA block while retaining the system's injectability at room temperature, sol–gel transition property at 37 °C and satisfactory gel stability. The copolymers with appropriate thermosensitive sol–gel transition property were selected for controlled release formulation of a model protein, lysozyme, and for evaluating the effect of copolymers on stability and activity of lysozyme. Moreover, the copolymers which showed better controlled release of lysozyme were examined for their *in vitro* biocompatibility using MTT assay and *in vivo* biocompatibility by histological examination of the skin tissue at injection sites.

## 2. Materials and methods

### 2.1. Materials

Monomethoxy poly(ethylene glycol) (mPEG550 and mPEG750) was purchased from Sigma (St. Louis, MO, USA). D,L-Lactide was obtained from TCI (Tokyo, Japan). Glycolide was bought from Maybridge (Cornwall, UK). Lysozyme (EC 3.2.1.17) from chicken egg white and *Micrococcus Lysodeikticus* (*Micrococcus luteus*) were purchased from Sigma (St. Louis, MO, USA). Micro-BCA protein assay reagent kit was purchased from Pierce (Rockford, IL, USA). Human embryonic kidney (HEK 293) cell line was obtained from American Type Culture Collection (Rockville, MD, USA). All other chemicals used were of analytical grade.

### 2.2. Copolymer synthesis

The triblock copolymer (mPEG–PLGA–mPEG) was synthesized by ring-opening polymerization following diblock condensation (Jeong et al., 1997; Singh et al., 2007a). Briefly, lactide and glycolide were polymerized onto mPEG chain to produce mPEG–PLGA diblocks which were then connected by the coupling agent isophorone diisocyanate (IPDI) to generate triblock mPEG–PLGA–mPEG copolymer. Fig. 1 shows the modified synthetic scheme of mPEG–PLGA–mPEG triblock copolymer. According to previously reported investigations, larger hydrophobic block sustained copolymer degradation over a longer duration, thereby resulting in the controlled release of incorporated therapeutic agents over a longer period (Chen et al., 2005a). Moreover, the ratio of the length of mPEG and PLGA blocks affected the thermosensitive sol–gel transition in a critical way (Packhaeuser et al., 2004). Thus, the lengths of both mPEG and PLGA blocks were elongated carefully, in order to find the copolymer with longest hydrophobic PLGA block length while conserving the copolymer/water system's injectability at room temperature, sol–gel transition and gel stability at 37 °C. The different block lengths of triblock copolymers were achieved by varying the reaction initiator mPEG (mPEG550 or mPEG 750) and adjusting the feeding ratio of mPEG to monomers (lactide and glycolide).

### 2.3. Copolymer characterization

#### 2.3.1. $^1H$ NMR analysis

$^1H$ NMR (Varian Unity, 300 MHz) was used to determine the structural composition of synthesized copolymers. All spectra were recorded at 25 °C in deuterated chloroform ($CDCl_3$) and tetramethylsilane (TMS) signal was taken as the zero chemical shift. Number average molecular weight ($M_n$) and the ratio of lactic acid (LA) to glycolic acid (GA) portions were calculated by integrating the signals pertaining to each monomer, such as the peaks from $CH_3$ of LA (at 1.55 ppm), $CH_3$ of mPEG end group (at 3.38 ppm), $CH_2$ of mPEG (at 3.65 ppm), $CH_2$ of GA (at 4.80 ppm), and CH of LA (at 5.20 ppm) (Jeong et al., 1999a).

#### 2.3.2. Gel permeation chromatography (GPC)

GPC was used to determine molecular weight distribution of synthesized copolymers. The measurement was carried out on a Waters 515 (Milford, MA) apparatus equipped with a refractive index detector and two Styragel® HR4E and HR5E columns (Milford, MA). The analyses were performed at 30 °C, using tetrahydrofuran (THF) as an eluant at a flow rate of 1 ml/min. Polystyrenes having molecular weight of 162–6,035,000 Da were used as standards for molecular weight calibration.