Journal of Controlled Release 171 (2013) 143–151

Contents lists available at ScienceDirect

Journal of Controlled Release

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

NANOMEDICINE

Temperature-modulated noncovalent interaction controllable complex for the long-term delivery of etanercept to treat rheumatoid arthritis

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ARTICLE INFO

Article history:
Received 2 April 2013
Accepted 7 July 2013
Available online 21 July 2013

Keywords:
Drug delivery
Electrostatic interactions
Etanercept
Noncovalent interactions
Rheumatoid arthritis

ABSTRACT

The clinical applications of etanercept (Enbrel), an emerging therapeutic protein for rheumatoid arthritis (RA), are limited by its instability and low bioavailability. In this study, a long-term and efficient therapeutic nanocomplex formulation for RA treatment was developed in the form of a temperature-modulated noncovalent interaction controllable (TMN) complex based on a temperature-sensitive amphiphilic polyelectrolyte (succinylated pullulan-g-oligo(L-lactide); SPL). The TMN complexes were prepared by simply mixing the negatively charged SPL copolymer and the positively charged etanercept *via* electrostatic interaction at 4 °C below the polymer's clouding temperature (CT), and the resulting complex demonstrated significantly improved salt and serum stability with increased hydrophobic interactions at temperatures (physiological condition, 37.5 °C) above the CT. An *in vitro* study of the bioactivity of etanercept indicated that the TMN complex improves the long-term stability of etanercept in an aqueous environment because of the exposure of the functional active site and the molecular chaperone-like effect of the hydrophobic copolymer. This formulation possessed prolonged *in vivo* pharmacokinetic parameters. In a collagen-induced arthritis RA rat model, we verified the outstanding therapeutic effect of the TMN complexes. These results imply that this approach would be widely applied to protein and peptide delivery systems.

1. Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by chronic, progressive inflammation and gradual joint destruction [1,2]. Although the root cause of RA remains unknown, it is a well-established fact that the proinflammatory cytokine tumour necrosis factor-a (TNF-α) plays a critical role in the pathogenesis of RA by orchestrating the inflammatory/immune-response in the synovium [3]. Accordingly, inhibiting the production and/or biological activity of TNF-α is considered a promising therapeutic approach. Most of the available therapeutic agents are therapeutic proteins such as etanercept (Enbrel), infliximab (Remicade), and adalimumab (Humira), which specifically bind to proinflammatory cytokines and other proteins [3,4]. Among these immunosuppressant proteins, etanercept, which is a genetically engineered fusion protein consisting of two identical chains of the recombinant human tumour necrosis factor receptor (TNFR) p75 monomer fused with the Fc domain of human IgG1, has the largest market due to safety and efficacy benefit [5]. However, despite their high activity and specificity, the therapeutic application of protein drugs suffers from poor biophysical stability (*i.e.*, high clearance rates) and low bioavailability [6,7]. These hurdles have led researchers to develop various vehicles that can carry proteins to prolong their half-life with reduced undesirable effects [8–11].

Recently, the polyelectrolyte (PE) complex has received a lot of attention in this field because it is formed easily *via* ionic interactions between charged proteins and counter-charged polymers in aqueous phase [12–14]. However, the lack of physical stability still remains unresolved; the polymer/protein complex dissociates during the fabrication process and readily releases proteins under physiological conditions (*i.e.*, physiological serum and salt concentrations) due to weak ionic interactions [15,16]. To overcome this limitation, several approaches have been reported to enhance the stability of PE complexes by covalent crosslinking of the core or shell [17,18]. However, the chemical reactions involved in the covalent interaction in the core or shell may induce side effects due to undesirable crosslinking [19].

Herein, we describe a sophisticated approach to stabilise etanercept and prolong its therapeutic effect against RA by using temperature-induced noncovalent interaction controllable (TMN) complex without covalent crosslinking. A temperature-sensitive amphiphilic polyelectrolyte was utilised to form PE complexes with etanercept, as well as to control hydrophobic interactions depending on the temperature (Scheme 1); this complex exhibited mainly electrostatic interactions between the negatively charged temperature-sensitive polymer and positively charged etanercept ($pI = 8.1$) at temperatures below the polymer's clouding temperature (CT). As temperature increased to over the polymer's CT (at physiological temperature, 37.5 °C), new noncovalent interactions (hydrophobic) are formed within

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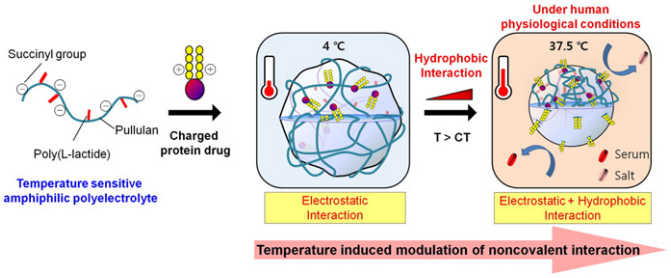
http://dx.doi.org/10.1016/j.jconrel.2013.07.012



Scheme 1. The TMN complexes were prepared by simply mixing the negatively charged temperature sensitive amphiphilic polyelectrolyte (SPL) and the positively charged etanercept *via* electrostatic interaction at temperature (4 °C) below the polymer's clouding temperature (CT), which can increase the hydrophobic interaction at human body temperature (37.5 °C). The increase of the hydrophobic interactions in TMN complexes results in size-shrinking and also causes significant enhancement in salt and serum stability.

the complex. Through this process, a substantial polymer/protein complex was finally formed without inter-particle aggregation *via* dual noncovalent interactions. Therefore, at human body temperature (37.5 °C), the stability of the polymer/etanercept complex against salt and serum could be significantly improved by increasing the hydrophobic interactions with the copolymer without any chemical crosslinking agent.

2. Materials and methods

2.1. Materials

Pullulan (MW: 100 kDa) was purchased from Hayashibara (Okayama, Japan). L-lactide (L-LA), succinic anhydride (SA), triethylamine (TEA), 4-dimethylaminopyridine (DMAP), dimethyl sulfoxide (DMSO), pullulan standard, type II collagen (CII), lipopolysaccharides (LPS) from *Escherichia coli* 0127:B8 and in/complete adjuvant were acquired from Sigma Chemical Company (St. Louis, USA). The fluorescent dye, Cy5.5 mono NHS ester, was obtained from Amersharm (Uppsala, Sweden), and the black hole quencher-3 (BHQ3) mono NHS ester was obtained from Biosearch Technologies (Novato, USA). Female Lewis rats that were 4 weeks old were purchased from Orient Bio (Kyungki-Do, South Korea). A TNF-α enzyme-linked immune-sorbent assay (ELISA) kit was obtained from eBioscience (San Diego, CA), and an etanercept ELISA kit was obtained from Matriks Biotek (Ankara, Turkiye).

2.2. Synthesis and characterization of SPL copolymer

Pullulan (1 g, 10 μmol) was suspended in DMSO (20 mL) with vigorous stirring at room temperature under a nitrogen atmosphere. SA (0.1 g, 1 mmol) and DMAP (0.07 g, 0.57 mmol) were added to the pullulan solution. The reaction mixture was stirred for 24 h and dialysed against distilled water (DIW) over 2 days using a dialysis membrane (molecular weight cut-off (MWCO), 10 kDa) to remove the solvent and unreacted small molecules. The resulting SPL0 polymer was lyophilised, and its chemical structure was confirmed by a 300 MHz Bruker NMR Spectrometer (Bruker, Germany). ^{1}H NMR (D_2O): succinic acid (–$\underline{CH_2}\underline{CH_2}$, 3.5–5.5 ppm, Fig. S2 (2)).

SPL was synthesised *via* ring-opening polymerization (ROP) of L-LA. In brief, L-LA (SPL1: 1.5 g, 10.41 mmol; SPL2: 1.75 g, 12.14 mmol; and SPL3: 2.0 g, 13.89 mmol) as a monomer and synthesised SPL0 (1 g, 10 μmol) as a macroinitiator were separately dissolved in DMSO. Two solutions (total 20 mL) were mixed at various feed ratios, and the reaction mixture was stirred at 70 °C under a nitrogen atmosphere. TEA (0.2 mL, 1.98 mmol) as a catalyst was added in the mixture. After 12 h, the mixture was dialyzed using a dialysis membrane (MWCO, 3.5 kDa) with DIW to remove unreacted L-LA and low molecular weight oligomers, and then SPL copolymer was lyophilized. The resulting polymer was characterized by ^{1}H NMR spectroscopy (Fig. S2) and GPC. ^{1}H NMR (D_2O): L-LA (–$\underline{CH_3}$, 1.5–1.7 ppm), internal methyl proton of polymerized L-LA (C–$\underline{H}$, 5.3 ppm), terminal methyl proton of polymerized L-LA (C–$\underline{H}$, 4.4 ppm).

The molecular weights of synthetic polymer were confirmed through gel permeation chromatography (GPC) using the following system: Waters 515 HPLC pump, Waters 410 differential refractometer, Shodex KF-804 columns. The eluent was a buffer solution (0.2 N $NaNO_3$, 0.01 N NO_2HPO_4 in ddH_2O), and the flow rate was 1 mL min^{-1}. The standard was calibrated with a pullulan standard. All the GPC curves of the SPL copolymers exhibit a mono peak pattern. The mole and weight contents of SA and L-LA in SPL copolymers were calculated by integrating the corresponding peaks, as summarised in Table 1.

To measure CTs of SPL copolymers, the cloud point measurement (turbidimetry) method was employed. Briefly, the SPL copolymers were dispersed (5 mg mL^{-1}) in DIW. The optical transmittances of these solutions were measured at 500 nm wavelength using UV–vis spectrometer (UV-1601, Shimadzu, Japan) with increasing solution temperatures (4–60 °C). At each temperature, the samples were stabilised for 30 min before measurements. Values for the CT of SPL copolymers were determined at a temperature with half of the optical transmittance between blow and above transitions.

CAC was measured using a reported method with some modification. A stock solution of Hoechst 33342 (1.4×10^{-3} M) in double-distilled water was prepared and stored at 5 °C until use. The Hoechst 33342 solution was mixed with solutions containing polymers at the concentrations of 1×10^{-4} mg mL^{-1} to 1.0 mg mL^{-1} at a certain temperature. The final concentration of Hoechst 33342 in each sample solution was 0.7×10^{-3} M. The resultant fluorescence was measured on a RF-5301PC (Shimadzu, Japan) with $\lambda_{ex} = 355$ nm and $\lambda_{em} = 457$ nm and the slit widths were ex = 3 nm and em = 3 nm, respectively.

2.3. Fluorescence labelling of SPL copolymer and etanercept

Cy5.5 mono NHS ester (0.2 mg, 0.2 μmol) was dissolved in DMSO (10 μL). Etanercept (10 mg, 0.07 μmol) was dissolved in sodium carbonate buffer (5 mL, 100 mM) at pH 9.3. The Cy5.5 solution was dropped in the etanercept solution and reacted with gentle stirring for 30 min at 4 °C. The solution was dialysed against DIW at 4 °C over

Table 1
Characterization of SPL copolymers.

	DS[a] (SA)	DS[b] (LA)	DP[c] (LA)	$M_{w,GPC}$[d] [×10^5 Da]	$M_{n,GPC}$[d] [×10^5 Da]	PDI[d]	$M_{n,^1H\ NMR}$[e] [×10^5 Da]	CT[f] [°C]	CAC[g] [mg mL^{-1}]	CAC[h] [mg mL^{-1}]
SPL1	0.09	0.43	1.61	2.28	2.20	1.04	2.19	48	0.16	0.11
SPL2	0.09	0.58	1.83	2.41	2.31	1.04	2.21	30	0.15	0.09
SPL3	0.09	0.92	1.95	2.53	2.21	1.04	2.31	22	0.11	0.04

[a]Degree of substitution of succinyl residue per 1 anhydroglucose unit of pullulan on the basis of the ^{1}H NMR results; [b]Degree of substitution of L-lactide per 1 anhydroglucose unit of pullulan on the basis of the ^{1}H NMR results; [c]Degree of polymerisation of L-lactide from 1 hydroxyl group of pullulan on the basis of the ^{1}H NMR results; [d]Number-averaged (M_n), weight-averaged molecular weight (M_w) and polydispersity index (PDI = M_w / M_n) were determined by GPC at 20 °C; [e]As determined by ^{1}H NMR; [f]The clouding temperature (CT) values of SPL copolymers were determined at the temperatures showing an optical transmittance (at 500 nm) of 50% by UV–vis spectrophotometer; As determined using a fluorescence dye (Hoechst 33342) technique at temperatures [g]below CT; [h]above CT.