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In situ depot formation of anti-HIV fusion-inhibitor peptide in recombinant protein polymer hydrogel

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ABSTRACT

Most peptide drugs have short half-lives, necessitating frequent injections that may induce skin sensitivity reactions; therefore, versatile prolonged-release delivery platforms are urgently needed. Here, we focused on an oxidatively and thermally responsive recombinant elastin-like polypeptide with periodic cysteine residues (cELP), which can rapidly and reversibly form a disulfide cross-linked network in which peptide can be physically incorporated. As a model for proof of concept, we used enfuvirtide, an antiretroviral fusion-inhibitor peptide approved for treatment of human immunodeficiency virus (HIV) infection. cELP was mixed with enfuvirtide and a small amount of hydrogen peroxide (to promote cross-linking), and the soluble mixture was injected subcutaneously. The oxidative cross-linking generates a network structure, causing the mixture to form a hydrogel *in situ* that serves as an enfuvirtide depot. We fabricated a series of enfuvirtide-containing hydrogels and examined their stability, enfuvirtide-releasing profile and anti-HIV potency *in vitro*. Among them, hydrophobic cELP hydrogel provided effective concentrations of enfuvirtide in blood of rats for up to 8 h, and the initial concentration peak was suppressed compared with that after injection of enfuvirtide alone. cELP hydrogels should be readily adaptable as platforms to provide effective depot systems for delivery of other anti-HIV peptides besides enfuvirtide.

Statement of Significance

In this paper, we present an anti-HIV peptide delivery system using oxidatively and thermally responsive polypeptides that contain multiple periodic cysteine residues as an injectable biomaterial capable of *in situ* self-gelation, and we demonstrate its utility as an injectable depot capable of sustained release of anti-HIV peptides. The novelty of this work stems from the platform employed to provide the depot encapsulating the peptide drugs (without chemical conjugation), which consists of rationally designed, genetically engineered polypeptides that enable the release rate of the peptide drugs to be precisely controlled.

1. Introduction

The application of effective antiretroviral therapy (ART) has transformed human immunodeficiency virus (HIV) infection into a long-term and manageable disorder [1]. Enfuvirtide (also known as Fuzeon, T-20 or DP178) is a potent antiretroviral fusion inhibitor composed of a linear sequence of 36 naturally occurring L-amino acid residues with an acetylated N-terminus and a carboxamide C-terminus, and prevents HIV from entering host cells [2–4]. Enfuvirtide interferes with an earlier stage of infection than standard ART regimens, and has been used in combination with other antiretroviral agents for the treatment of HIV-infected individuals and AIDS patients with multidrug-resistant HIV infections that no longer respond to standard ART. Despite its therapeutic efficacy, the clinical use of enfuvirtide is limited, as the peptide is rapidly cleared from the circulation, principally by degradation in the liver and renal clearance, resulting in a short plasma half-life of approximately 2 h [2]. To maintain an effective blood concentration, a twice-daily subcutaneous (*s.c.*) injection schedule has been established clinically, but due to the frequency of administration, skin

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sensitivity reaction side effects often occur at the injection sites [5,6], and this eventually decreases patient compliance.

Significant effort has hence been expended to develop longer-acting analogs of enfuvirtide with the goal of generating a drug that will provide a well-sustained, flat concentration-versus-time profile in the therapeutic range. Such an agent would not only reduce the necessary number of injections, but also prevent "peak-and-valley" fluctuations associated with *s.c.* injections of short-acting analogs that provide a therapeutically effective drug concentration for only short time and may also have undesired side effects, including development of drug-resistant strains of HIV. Several groups have proposed the attachment of enfuvirtide to carrier molecules such as polyethylene glycol (PEG) [7], xten [8], sialyllactose [9], pentasaccharide [10], dihydrosphingosine [11], serum albumin [12], or Fc fragments [13] as a strategy to obtain prolonged circulation. However, these chemical modifications require site-specific covalent bond formation, which generally has a low yield and is often difficult to scale-up. After enfuvirtide, second- and third-generation derivatives with increased antiviral potency were developed [14,15], and many synthetic peptides that interact with HIV *in vitro* [16,17] are candidates for stopping the lifecycle of HIV via different mechanisms from those of standard drugs used in ART. A general methodology for *in vivo* delivery without the need for chemical conjugation of individual peptides is therefore needed. Kapoor et al. approached this issue by *in situ* formation of enfuvirtide-containing implants using poly (D,L-lactide-co-glycolide) with organic solvents [18].

Elastin-like polypeptides (ELPs) are a class of artificial polypeptides composed of the pentameric repeat Val-Pro-Gly-Xaa-Gly (where Xaa is any amino acid except Pro). ELPs are increasingly utilized for many biomedical applications, as they are non-cytotoxic, biodegradable, and show good pharmacokinetics [19]. We are interested in the design of *in situ* depot using ELPs for peptide drugs that need drug delivery system, especially anti-HIV peptides. Herein we describe the design and characterization of injectable hydrogels as a platform for *in situ* formation of subcutaneous drug depots to provide sustained release of anti-HIV peptides. The central hypothesis underlying this design is that hydrogels can entrap a sufficient amount of peptide to form a drug depot, which would be slowly degraded, resulting in prolonged release of active peptide into the circulation. To validate this idea, we selected enfuvirtide as a model anti-HIV peptide. In this system, enfuvirtide is physically mixed with a recombinant elastin-like polypeptide that incorporates periodic cysteine (Cys) residues (cELP), and then with a small amount of hydrogen peroxide that serves as an oxidant to rapidly form a disulfide cross-linked network [20]. The preparation remains in a soluble, injectable state, but undergoes gelation and phase transition (dehydration) between room and body temperature. Therefore, a hydrogel is formed *in situ* after *s.c.* injection. This hydrogel depot is slowly degraded, resulting in prolonged release of enfuvirtide. Our present results confirm the validity of this strategy, and we show that a single *s.c.* injection of enfuvirtide-containing hydrophobic cELP in rats forms a hydrogel depot that provides prolonged anti-HIV activity as compared with injection of the naked peptide. We believe this cELP hydrogel platform should be readily adaptable to provide novel delivery systems for a variety of anti-HIV peptides besides enfuvirtide.

2. Materials and methods

2.1. Materials

Escherichia coli (*E. coli*) EB5alpha and BL21 (DE3) cells were purchased from EdgeBio (Gaithersburg, MD) and EMD Chemicals (San Diego, CA), respectively. All cultures were grown in Terrific Broth (TB) Dry medium from MO Bio Laboratories (Carlsbad, CA). Tris (2-carboxyethyl)phosphine (TCEP) hydrochloride and Slide-A-Lyzer dialysis cassette were purchased from Pierce Biotechnology (Rockford, IL). Polymyxin B sulfate was purchased from EMD Chemicals. Enfuvirtide was purchased from ProSpec-Tany TechnoGene (Ness Ziona, Israel). Hydrogen peroxide (H_2O_2; 30 wt% solution), sodium sulfite (Na_2SO_3), and elastase (from porcine pancreas) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Dojindo Laboratories (Kumamoto, Japan). Copper chloride, polyethyleneimine (PEI; 50% (w/v) solution), azidothymidine (AZT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide (MTT), and RPMI 1640 medium were purchased from Sigma-Aldrich (St. Louis, MO). Sodium curdlan sulfate (79 kDa) was from Ajinomoto (Tokyo, Japan). Precast SDS-PAGE 5–15% Tris-HCl gradient gel was obtained from D.R.C. (Tokyo, Japan). A human T-cell leukemia virus type I (HTLV-I) positive T-cell line, MT-4 [21] was used for evaluating anti-HIV activity. MOLT-4 cells [22], a lymphoblastoid T-cell line, were used for obtaining HIV-1_{IIIB} (an X4 HIV-1 stain). HIV-1_{IIIB} was prepared from culture supernatant of persistently infected MOLT-4/HIV-1_{IIIB} cells and stored at −80 °C, and the titer of the virus stock was determined as 50% tissue culture infectious doses ($TCID_{50}$) using MT-4 cells [23]. MT-4 cells and MOLT-4 cells were cultured at 37 °C a humidified atmosphere of 5% CO_2 in air, using RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 100 units mL^{-1} of penicillin, and 100 μg mL^{-1} of streptomycin (Gibco Life Technologies, Grand Island, NY).

2.2. Nomenclature

ELPs are described with the nomenclature ELP[$A_xV_yC_z$-*n*], where *n* refers to the number of pentapeptide repeats and x, y, and z refer to the relative fractions of alanine, valine, and cysteine in the guest residue position along the length of the polypeptide, respectively. The polypeptides used in this study have the composition ELP [$A_{14}V_1C_1$-160] (cELP1) and ELP[$V_{15}C_1$-160] (cELP2). ELP[V-160] (ELP_{ctrl}), which does not contain Cys residues, was used as a control polypeptide. Cys-containing ELPs are denoted as cELP throughout the paper to distinguish them from general ELPs (for full sequence details, see Fig. S1).

2.3. Construction of ELPs

ELPs were designed and synthesized as described previously [20]. Briefly, BL21(DE3) *E. coli* cells were transformed with recombinant pET-24a(+) vector containing ELP genes. The synthesis of ELPs was based on constitutive expression from the leaky T7 promoter in the host *E. coli* cells. 1 L of TB media supplemented with 50 μg mL^{-1} kanamycin was inoculated with 35 mL of an overnight culture, and incubated overnight at 37 °C on an orbital shaker. The *E. coli* cells were harvested by centrifugation at 2500*g* for 10 min and then re-suspended in phosphate-buffered saline (PBS) and lysed by ultrasonication. To precipitate genomic DNA with insoluble cell debris, the lysate was supplemented with polyethyleneimine (at 1.2 wt%) and centrifuged at 16,000*g* at 4 °C. Each cELP was purified from the soluble fraction of cell lysate by 5 rounds of inverse transition cycling (ITC) [24] with TCEP solution (pH 7.0) and re-suspended in 20 mM TCEP (pH 7.0) supplemented with 1500 units mL^{-1} polymyxin B sulfate. ELP_{ctrl} was also purified by means of 5 rounds of ITC with PBS instead of TCEP. The purified ELPs were dialyzed at 4 °C for 16 h against PBS using 10-kDa molecular weight cut-off Slide-A-Lyzer dialysis cassettes. The purity and molecular weight (MW) of ELPs were examined by SDS-PAGE. The gels were stained with 0.5 M copper chloride, and ELP concentration was determined spectrophotometrically using the

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