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Single chain Fc-dimer-human growth hormone fusion protein for improved drug delivery

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ABSTRACT

Fc fusion protein technology has been successfully used to generate long-acting forms of several protein therapeutics. In this study, a novel Fc-based drug carrier, single chain Fc-dimer (sc(Fc)$_2$), was designed to contain two Fc domains recombinantly linked via a flexible linker. Since the Fc dimeric structure is maintained through the flexible linker, the hinge region was omitted to further stabilize it against proteolysis and reduce FcγR-related effector functions. The resultant sc(Fc)$_2$ candidate preserved the neonatal Fc receptor (FcRn) binding. sc(Fc)$_2$-mediated delivery was then evaluated using a therapeutic protein with a short plasma half-life, human growth hormone (hGH), as the protein drug cargo. This novel carrier protein showed a prolonged *in vivo* half-life and increased hGH-mediated bioactivity compared to the traditional Fc-based drug carrier. sc(Fc)$_2$ technology has the potential to greatly advance and expand the use of Fc-technology for improving the pharmacokinetics and bioactivity of protein therapeutics.

1. Introduction

The large foundation of knowledge of immunoglobulin G (IgG) molecules has promoted development and optimization of protein-based therapeutics and delivery systems, including monoclonal antibodies (mAbs), immunotoxins, antibody-drug conjugates, and Fc-fusion proteins [1]. Fc-fusion proteins, due to binding of the IgG Fc domain to the neonatal Fc receptor (FcRn), are involved in the recycling and transcytosis pathways of IgG [2]. The Fc domain of IgG binds FcRn with high affinity at an acidic pH (<6.5), but with negligible binding affinity at physiological pH (7.4) [3]. In cells with a slightly acidic extracellular pH, such as the small intestines, IgG binds to FcRn at the cell surface followed by transcytosis of IgG from the apical to the basolateral surface [4–6]. The subsequent exposure to the pH of blood, which is approximately 7.4, allows for the dissociation and release of IgG into circulation. For cells with a neutral extracellular pH, it is generally thought that IgG is internalized by fluid-phase pinocytosis, binds to FcRn in the acidified endosome, and is then either recycled or transcytosed [7]. By interacting with FcRn through the recycling and/or transcytosis pathway, IgG can avoid degradation in lysosomes, resulting in its long plasma half-life. The strategy of engineering Fc conjugates takes advantage of this characteristic to prolong the plasma half-life of protein drugs [7].

To date, there are nine FDA-approved Fc-fusion proteins, and many others are at different stages of clinical and preclinical development. A majority of Fc-fusion protein drugs consist of a protein drug linked to the N-terminal of an Fc domain that forms a drug-Fc homodimer ("(drug-Fc)$_2$") along with drug-Fc monomer impurities (Fig. 1). In the (drug-Fc)$_2$ homodimer configuration, the protein drug domains are adjacent to each other, often leading to their physical instability and/or decreased bioactivity [8,9]. Further, many large protein drugs are not suitable candidates as they cannot be stably expressed [10]. In order to overcome these disadvantages, "Monomeric" Fc-fusion proteins (Fig. 1), containing a protein drug

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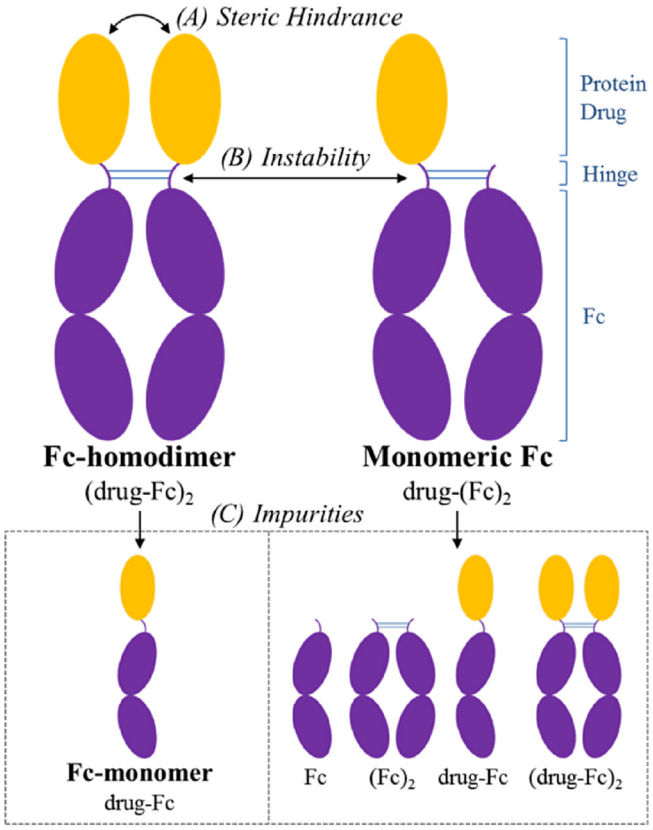

Fig. 1. Schematic structures of current Fc-fusion protein technology and their disadvantages. A majority of FDA-approved Fc-fusion proteins exist as a Fc-homodimer. **(A)** In this configuration, steric hindrance between the protein domains leads to physical instability, decreased bioactivity and/or limitations in the size of protein that can be accommodated. Monomeric Fc fusion proteins have been recently developed to overcome these limitations. Both Fc-homodimers and Monomeric Fc fusion proteins are **(B)** susceptible to instability in the hinge region via protease digestion and disulfide reduction, and **(C)** generate several impurities during recombinant production.

linked to only one of the two Fc domains ("drug-$(Fc)_2$") have recently been tested and clinically approved (eg. Alprolix® and Eloctate®). Studies have shown that these Monomeric Fc-fusion proteins have improved half-lives and/or bioactivity compared to their homodimeric counterparts [8]. However, their main limitation is production, which requires dual expression plasmids containing the drug-Fc and the Fc sequences. This production protocol generates a mixture of multiple fusion products including (drug-$Fc)_2$, drug-$(Fc)_2$ and $(Fc)_2$, creating issues of impurity. Further, formation of homodimers (i.e. (drug-$Fc)_2$ and $(Fc)_2$) are favored over the Monomeric drug-$(Fc)_2$ products, resulting in low production yields and instability [11]. Due to these limitations, this promising technology cannot be applied to all protein drugs. Current Fc-fusion proteins maintain the hinge region sequence of IgG to link the two Fc domains via disulfide bonds (Fig. 1). Other than linking two Fc domains, this region is not important for Fc function but introduces potential instability due to disulfide reduction, and also via enzymatic degradation at several protease cleavage sites present in the hinge region [12]. Additionally, the lower hinge of IgG Fc plays a crucial role in its binding to Fcγ receptors (FcγR), initiating effector functions that are out of the designed mechanism of action [13,14].

In this study, we have designed and evaluated a novel type of Fc fusion protein by using a long, flexible glycine-serine (GS) linker [15] to link two Fc chains, with the hinge sequence removed, to create a single chain Fc-dimer, sc$(Fc)_2$. A flexible, rather than a rigid, linker was used in this study to allow the two Fc domains to interact properly with each other. This type of linker is utilized in a variety of fusion proteins, including the well-established single chain antigen binding proteins (i.e. scFv) [15,16]. The use of this novel design allows for the advantages of a Monomeric Fc-fusion protein, without the issues of production impurities and requirement of the hinge region. In this study, human growth hormone (hGH) was linked to sc$(Fc)_2$ to evaluate the protein drug delivery properties of this novel carrier protein. hGH deficiency is associated with several clinical indications, including short stature, Turner syndrome, chronic kidney disease, HIV-associated wasting and abnormal metabolism [17]. hGH has a very short plasma half-life of 3.4 h after subcutaneous injection, and 0.36 h after intravenous injection [18]. Therefore, current treatment of hGH deficiency is limited to needle injection of hGH several times a week, which is not favored by patients, especially children and seniors [17]. hGH-sc$(Fc)_2$ fusion protein was then evaluated for its hGH-mediated bioactivity and pharmacokinetic properties. The evaluation of this novel Fc carrier protein could also provide insights to mechanistic studies of Fc-FcRn interaction and future application to other therapeutic peptides or proteins.

2. Materials and methods

2.1. Cell culture

The human embryonic kidney cell line HEK293 and human colon carcinoma cell line T84 were purchased from ATCC (Manassas, VA), and Nb2 cells derived from rat T lymphoma cells were purchased from Sigma (St. Louis, MO). Cell culture media were all from Mediatech (Manassas, VA). HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 2.5 mM L-glutamine supplemented with 10% fetal bovine serum (FBS) and 50 units of penicillin/50 μg streptomycin. T84 cells were cultured in 1:1 mixture of Ham's F12 medium and DMEM with 2.5 mM L-glutamine, 10% FBS, and 50 units of penicillin/50 μg streptomycin. Nb2 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine, 10% FBS, 10% horse serum, 50 units of penicillin/50 μg streptomycin, and 50 μM 2-mercaptoethanol. All cell lines were maintained in a humidified incubator at 37 °C with 5% CO_2.

2.2. Plasmid construction

2.2.1. pcDNA3.1+_sc$(Fc)_2$

A series of plasmids encoding sc$(Fc)_2$ with different linker lengths, $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_n$ ("$(G_4S)_n$") with n = 8–14, were constructed using pcDNA3.1+ (Invitrogen, Carlsbad, CA) as the vector and the commercial plasmid pFUSE-hIgG1-Fc2 (Invivogen, San Diego, CA) as the template of Fc sequence (Fig. 2A). The pFUSE-hIgG1-Fc2 contains the DNA sequence of CH2 and CH3 domains of wild-type hIgG1 with IL2 secretion sequence and a truncated hinge sequence at the beginning of the CH2 domain. The truncated hinge sequence was removed during sub-cloning.

2.2.2. pcDNA3.1+_hGH-sc$(Fc)_2$

The pcDNA3.1+_sc$(Fc)_2$ was double digested with *Hin*dIII and *Eco*RI restriction enzymes (New England Biolabs, Ipswich, MA) to replace the IL2 secretion signal sequence with the hGH sequence, which has its own secretion signal, allowing for the expressed fusion protein to be collected from the culture medium.

2.2.3. pcDNA3.1+_hGH-Fc

The pcDNA3.1+ expression vector harboring hGH-transferrin [17] was double digested with *Xho*I and *Xba*I restriction enzymes (New England Biolabs) to replace the transferrin fragment with the