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Access to site-specific Fc–cRGD peptide conjugates through streamlined expressed protein ligation†

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Abstract

An ideal drug should be highly effective, non-toxic and be delivered by a convenient and painless single dose. We are still far from such optimal treatment but peptides, with their high target selectivity and low toxicity profiles, provide a very attractive platform from which to strive towards it. One of the major limitations of peptide drugs is their high clearance rates, which limit dosage regimen options. Conjugation to antibody Fc domains is a viable strategy to improve peptide stability by increasing their hydrodynamic radius and hijacking the Fc recycling pathway. We report the use of a split-intein based semi-synthetic approach to site-specifically conjugate a synthetic integrin binding peptide to an Fc domain. The strategy described here allows conjugating synthetic peptides to Fc domains, which is not possible *via* genetic methods, fully maintaining the ability of both the Fc domain and the bioactive peptide to interact with their binding partners.

Introduction

Peptides are pharmacologically very attractive agents due to their high selectivity, potency, low toxicity, chemical diversity and amenability to rational design.^{1–3} These biochemical and biophysical advantages are reflected in the high success rates of peptide drugs during clinical development.⁴ There currently are more than a hundred peptide based drugs in the market, including blockbusters such as the immunomodulator Copaxone for multiple sclerosis, and gonadotropin-releasing hormone receptor agonists Lupron and Zoladex, for cancer and endometriosis.³ However, peptides have traditionally been considered unsuitable drug candidates due to poor oral bioavailability and low serum half-lives. Recent advances in painless and patient friendly delivery systems such as needle-free injection, are changing the public and industry perception on non-orally deliverable drugs, eliminating this traditional barrier for peptides.² Additionally several methods have been developed to improve peptide stability in circulation, including chemical modification to increase hydrodynamic radius^{5,6} or stability to proteases.^{1,3} Conjugation to antibody Fc fragments^{7–10} has also been

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successfully used to improve peptide clearance rates, by hijacking the neonatal Fc receptor (FcRn) recycling process, responsible for the long circulating half-life of type G immunoglobulins (IgG).^{11,12}

Traditionally genetic⁸ methods have been leveraged to append peptides onto Fcs, or full IgGs, to benefit from the FcRn pathway and concurrently increase their hydrodynamic radius. Genetic fusions are the strategy of choice for peptides that can be genetically encoded and recombinantly produced. Several Fc–polypeptide fusion proteins with long half-lives, including the approved drugs etanercept and romiplostim, as well as numerous advanced clinical candidates illustrate the usefulness of the concept.

Introduction of unnatural modifications (cyclizations, non-proteinogenic amino acids) is an alternative and often employed strategy in the discovery and development of peptide based drugs. These peptides need to be prepared by chemical synthesis and thus are not amenable to genetic fusion to Fc, and chemical conjugation methods have to be employed. Examples of such peptides are cyclic synthetic peptides derived from the RGD motif, which have been identified as potent integrin antagonists and have been shown to have anti-angiogenic and anti-tumor activities,¹³ in pre-clinical¹⁴ and clinical studies.¹⁵ Moreover, cyclic RGD peptides show promise as targeting agents.¹³ Despite their great potential, RGD peptides suffer from some of the common limitations associated with other peptides, including short serum half-lives, and so chemical conjugation to immunoglobulins has been proposed as a viable strategy to improve their potential as therapeutic agents.^{16,17}

Site-specific conjugation technologies are currently being developed as alternatives to traditional bioconjugation approaches, to yield homogeneous Fc–peptide conjugates,^{17–19} which have more predictable and controllable properties. Several site-specific conjugation methods are currently available, including: (i) incorporation of unnatural amino acids,²⁰ (ii) enzymatic modification of amino acid side chains,¹⁹ and (iii) chemical-²¹ or (iv) enzymatic-based ligation technologies.²² All these strategies are additions to the modern bioconjugation toolbox, from which the most appropriate need to be selected and tailored for each particular target molecule or application.²³ Expressed Protein Ligation (EPL)²⁴ is one of the most widely used site-specific modification technologies, and has been applied to a broad variety of proteins,²¹ including antibodies^{25,26} and, recently, antibody fragments.¹⁸ However, commonly used inteins to generate the required recombinant α -thioesters suffer limitations due to slow reaction kinetics, as well as premature hydrolysis during protein expression and purification. An EPL variant, termed Streamlined Expressed Protein Ligation (SEPL)²⁷ was recently developed to address these problems, and facilitate the chemical modification of large, challenging proteins such as antibodies and antibody fragments. SEPL relies on the use of ultrafast split-inteins²⁸ as latent protein α -thioesters, for their subsequent ligation to N-terminal Cys containing peptides.²⁷ The use of ultrafast split inteins reduces overall reaction times and eliminates the risk of premature hydrolysis during recombinant expression and purification of the intein tagged intermediates. To the best of our knowledge the conjugation of an Fc-IgG to a biologically active peptide, under control of the hydrolysis byproduct has not been reported.

Results and discussion

Semi-synthesis of cRGD peptide–Fc conjugates

We decided to use SEPL to conjugate an integrin binding RGD peptide to a human Fc domain (Fig. 1) as a first step towards the development of a generally applicable, scalable method to prepare homogeneous Fc–peptide conjugates for drug discovery and development. A critical success factor for our project was to minimize formation of the hydrolysis side product Fc–OH, which would be difficult to separate from the desired Fc–RGD conjugate. Amongst the many RGD peptide variants we selected the cyclic peptide *cyclo*(Lys–Arg–Gly–Asp–D–Phe), for its tight integrin α V β 3 binding^{16,29} and its tolerance to Lys side chain modification, which provided a suitable site to incorporate the required α -Cys residue for ligation (Cys–cRGD, Fig. 1). A mammalian expression system (CHO cells) was employed to produce the Fc to avoid potential challenges with refold of the Fc-fragment, observed when employing *E. coli* based expression systems.

Previous work has shown that expression yields of intein fusions can vary greatly depending on the identity of the intein being used.^{27,28} Hence, several ultrafast N-terminal DnaE intein fragments were fused to the Fc domain of human IgG1 C-termini, to identify which one of them produced acceptable Fc-intein titers. Western blot analysis of cell supernatants from transiently transfected CHO cells, cultured in suspension, showed that the N-terminal fragment of the DnaE intein from *Anabaena variabilis* (AvaN) was the one that produced the highest amounts of Fc-intein fusion (ESI Fig. S1[†]). Constructs were also optimized to ensure clean removal of the leader IL2 signal sequence and generation of homogeneous secreted Fc-intein fusions (ESI Fig. S2[†]). Importantly, AvaN fusion did not affect the Fc glycosylation pattern based on RP-HPLC and MS analysis (ESI Fig. S3[†]). The selected Fc–AvaN construct (Fig. 1) was recombinantly expressed in transiently transfected CHO-S cells and purified over Protein G prior to its chemical modification.

Protein thiolysis from N-intein fusions, and generation of α -thioesters can be performed, as previously described, using engineered IntC fragments,²⁷ in solution or on a solid support. IntC immobilization onto a solid support provides an efficient strategy to simultaneously purify the target protein and generate its C-terminal thioester. However, considering Fc fusions could be conveniently purified using Protein G, we decided to perform thiolysis and ligation reactions in solution, to facilitate monitoring and optimization of reaction conditions in a homogeneous phase.

Thiolysis and peptide ligation were carried out in one-pot, upon mixing the purified Fc–AvaN protein, with the engineered IntC fragment and an N-terminal Cys containing peptide (Fig. 1). Initial reaction tests were performed with the model N-terminal Cys peptide H-Cys–Gly–Lys(fluorescein)–OH, (CGK(FI)). Thiolysis from the AvaN intein, and ligation to the *in situ* generated α -thioester were monitored by SDSPAGE (Fig. 2A and ESI Fig. S4[†]) and RP-HPLC (Fig. 2B), respectively. Prior to RP-HPLC analysis, and to facilitate the resolution of ligated and hydrolyzed species, samples were deglycosylated and fully reduced to generate

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monomeric C_H2–C_H3 chains. Intein thiolysis proceeded with yields over 85%, while a strong dependence on the N-terminal Cys peptide concentration was observed for ligation yields, as expected for an NCL reaction (Fig. 2, Table 1 and ESI Fig. S6[†]). Thiolysis/ligation conditions were thus carefully monitored by RP-HPLC and MS, and optimized to ensure maximum conjugation yields and minimize thioester hydrolysis. MS analysis of the reaction mixture under optimal conditions confirmed the absence of detectable amounts of hydrolyzed C_H2–C_H3 chains (Fig. 2 and ESI Fig. S5[†]). The conjugates were confirmed to be Fc dimers by size exclusion chromatography, ESI-MS and SDS-PAGE analysis under reducing and non-reducing conditions (ESI Fig. S7 and S8[†]).

Identified optimal reaction conditions were used to ligate the Cys-cRGD peptide to the Fc (Fig. 2C). Ligation proceeded with yields over 95% (based on RP-HPLC of fully reduced samples) and the formation of the desired product was confirmed by MS analysis (Fig. 2D and ESI Fig. S9[†]). Excess reagents were removed by dialysis and the Fc–cRGD conjugate was purified by size exclusion chromatography under native, non-reducing conditions. The Fc–cRGD conjugate elution volume was consistent with the presence of an intact Fc dimer, and SDS-PAGE analysis under reducing and non-reducing conditions confirmed the conjugate was a disulfide containing dimer with the expected apparent MW (Fig. 3B). Furthermore, LC-MS analysis of the purified Fc–cRGD conjugate confirmed the expected MW for an Fc dimer with the expected glycosylation pattern (Fig. 3C, D and ESI Fig. S11[†]). Importantly, no heterodimeric Fc species, in which only one of the two chains were modified with the cRGD peptide and the other was the Fc–OH, were detected by MS. This is a direct consequence of the high ligation yields and minimal hydrolysis levels, and a key feature in this efficient process to prepare Fc–peptide conjugates. Since expressed protein ligation introduces cysteines at the conjugation site, potential concerns regarding long-term stability of the resulting conjugates have to be addressed. Unpaired thiols have been implicated in protein aggregation,^{30,31} and therefore it was important to confirm complete pairing of the cysteines of the purified construct. Treatment of the conjugate with iodoacetic acid under denaturing conditions did not result in any change in the MS data as compared to the untreated material, thereby clearly demonstrating full pairing of the newly introduced cysteines (ESI Fig. S12[†]). Typical overall yields of 5–6 mg L^{–1} expression media of purified conjugate were obtained from transiently transfected CHO cells. Importantly, the overall yield from the recombinantly expressed Fc–AvaN to the final purified conjugate was 42%.

Fc–cRGD conjugates bind the Fc–Rn and integrin αVβ3

We next set to test if the Fc–cRGD obtained by SEPL had the expected biochemical properties for a conjugate containing an Fc fragment and an integrin binding cRGD peptide. First, we measured binding of the modified Fc–cRGD to the Fc–Rn receptor using Surface Plasmon Resonance (SPR). Fc–cRGD was able to bind to immobilized Fc–Rn at pH 6.0 with a K_D of 97 ± 1 nM, comparable to that of a non-modified Fc (Fc–OH) recombinantly produced in *E. coli* (Fig. 4). K_D values were also in good agreement with those previously reported.³² Importantly, Fc–cRGD binding to the Fc–Rn displayed the expected pH dependence and no binding was observed at pH 7.4, which is a key feature for efficient Fc recycling *via* the Fc–Rn pathway. Functionality of the cRGD peptide conjugated to the Fc was also confirmed by SPR using immobilized integrin αVβ3. The Fc–cRGD conjugate

bound to integrin $\alpha V\beta 3$ with a K_D of 880 ± 30 nM, which is in good agreement with values previously described in the literature.^{29,33}

Conclusions

In conclusion, we have shown here that SEPL is an efficient method to generate Fc-peptide conjugates. Thiolytic/ligation reactions proceed in good yields and the use of ultrafast, high expressing split-inteins, completely eliminates the risk of premature intein hydrolysis during recombinant expression and purification and reduces overall reaction and processing times. This strategy allowed us to generate Fc-peptide conjugate dimers with no detectable heterogeneity due to hydrolysis. This is, to the best of our knowledge the first report of the use of split inteins to generate Fc α -thioesters for site-specific conjugation of bioactive peptides. The newly introduced cysteines at the C-terminus of the Fc form a disulfide bridge, alleviating potential concerns about long-term instability and disulfide scrambling due to unpaired cysteines. Most importantly, dimeric Fc-peptide conjugates obtained with this strategy fully retain Fc and peptide biochemical properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Streamlined expressed protein ligation to generate Fc-cRGD conjugates. (A) Scheme of the Fc-IntN constructs used in this study. The C-terminus of the C_H2 and C_H3 domains of human IgG1 were fused to the N-terminus of DnaE N-intein fragments (IntN). The leader sequence of interleukin 2 (IL2) was used to promote secretion from the production cell line. (B) SEPL scheme. Recombinantly produced Fc-IntN fusions are mixed with an engineered IntC fragment and an N-terminal Cys containing peptide (Cys-cRGD) in the presence of MESNa to yield Fc-cRGD. (C) Amino acid sequence of the engineered IntC peptide used in this study.

Fig. 2.

One pot thiolysis/ligation reactions. (A) Coomassie stained SDSPAGE analysis, under reducing conditions, of one-pot thiolysis/ligation of $250 \mu\text{g mL}^{-1}$ Fc-AvaN, $14 \mu\text{M}$ IntC and 0.5 mM CGK(FI) in phosphate buffer containing 1 mM EDTA, 1 mM TCEP and 100 mM MESNa at pH 7.2 for 24 h at r.t. (B) RP-HPLC analysis (detection at 280 nm) of thiolysis/ligation reactions after 24 h at different CGK(FI) peptide concentrations. Samples were deglycosylated and fully reduced prior to RP-HPLC analysis. (C) RP-HPLC analysis (detection at 280 nm) of one-pot thiolysis/ligation of $500 \mu\text{g mL}^{-1}$ Fc-AvaN and 0.5 mM Cys-cRGD after 24 h at r.t. Sample was deglycosylated and fully reduced prior to RP-HPLC. (D) ESI-MS analysis of deglycosylated and fully reduced C_H2C_H3-cRGD. The whole C_H2C_H3-cRGD labeled RP-HPLC peak was analyzed to ensure detection of C_H2C_H3-cRGD as well as C_H2C_H3-OH.

Fig. 3.

Purification and analysis of Fc-cRGD dimer. (A) Size exclusion chromatography of reaction mixture. (B) Coomassie stained SDS-PAGE analysis of purified Fc-cRGD conjugate under reducing (+βME) and non-reducing conditions (-βME). (C) ESI-MS analysis of deglycosylated non-reduced Fc-cRGD conjugate. (D) ESI-MS analysis of intact Fc-cRGD conjugate. Major glycoform had a MW in agreement with an Fc dimer containing two N-linked G0 glycans.

Fig. 4. Binding of Fc-cRGD and Fc-OH to the FcRn and integrin $\alpha V\beta 3$. (A) Fc-cRGD and Fc-OH binding curves (right) to immobilized FcRn at pH 6.0, obtained by single exponential fitting of SPR sensorgrams (left). No binding was observed at pH 7.4. (B) Fc-cRGD (right) and Fc-OH (left) binding sensorgrams to $\alpha V\beta 3$.

Table 1Ligation yields^a

[Cys-peptide] (μM)	Ligation yield (%)
12.5	26
25	53
50	75
100	90
500	>95

^aLigation yields estimated by RP-HPLC analysis of thiolysis/ligation reactions after complete deglycosylation and reduction of Fc related species.

Ligation reactions were set up using 250 μg mL⁻¹ Fc-AvaN in phosphate buffer saline containing 1 mM EDTA, 1 mM TCEP, 14 μM of IntC peptide at pH 7.2 and the indicated concentration of the N-terminal Cys containing peptide CGK(FI) and allowed to proceed for 24 h. Ligation yields were calculated by integrating the intensities of peaks corresponding to the ligated and un-ligated Fc species.