Interferon dimers: IFN-PEG-IFN

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Abstract

Increasingly complex proteins can be made by a recombinant-chemical approach where proteins that can be made easily can be combined by site-specific chemical conjugation to form multifunctional or more active protein therapeutics. Protein dimers may display increased avidity for cell surface receptors. The increased size of protein dimers may also increase circulation times. Cytokines bind to cell surface receptors that dimerise, so much of the solvent accessible surface of a cytokine is involved in binding to its target. Interferon (IFN) homo-dimers (IFN-PEG-IFN) were prepared by two methods: site-specific *bis*-alkylation conjugation of PEG to the two thiols of a native disulfide or to two imidazoles on a histidine tag of two His_8-tagged IFN (His_8IFN). Several control conjugates were also prepared to assess the relative activity of these IFN homo-dimers. The His_8IFN-PEG_{20}-His_8IFN obtained by histidine-specific conjugation displayed marginally greater *in vitro* antiviral activity compared to the IFN-PEG_{20}-IFN homo-dimer obtained by disulfide re-bridging conjugation. This result is consistent with previous observations in which enhanced retention of activity was made possible by conjugation to an N-terminal His-tag on the IFN. Comparison of the antiviral and antiproliferative activities of the two IFN homo-dimers prepared by disulfide re-bridging conjugation indicated that IFN-PEG_{10}-IFN was more biologically active than IFN-PEG_{20}-IFN. This result suggests that the size of PEG may influence the antiviral activity of IFN-PEG-IFN homo-dimers.

Introduction

As therapeutic proteins become more complex there is an opportunity to consider approaches that utilize site-specific conjugation technologies to link proteins that are easily made. Such a hybrid recombinant-chemical approach could in principle be used in a modular way to make a wide range of multifunctional proteins that would not be practical and cost-effective to make by recombinant technologies alone, especially in the early stage of development. An exclusively recombinant approach to make multifunctional therapeutic proteins requires that a polypeptide sequence must be used to link the two or more proteins together. As a non-natural sequence, the linking region may be immunogenic, so polypeptide linkers must be both functional and non-antigenic. As proteins become more complex, there is also often an increased propensity for aggregation and degradation during manufacture and storage[1-2].

Many proteins are structurally and functionally complex and form dimers[3]. Interferon (IFN) is a cytokine that was first described as an antiviral agent[4]. Type 1 IFNs act on a target cell (not the virus) to inhibit replication and the lifecycle of a wide variety of viruses, thus conferring a state of resistance within the cell to viral infection[5]. There are a number of pharmaceutical forms of Type I IFNs, namely Infergen®, Intron A®, PegIntron® and Pegasys® for the treatment of viral diseases such as Hepatitis B and Hepatitis C[6]. IFN α-2b in the presence of zinc forms dimers that have been found to have similar crystal structures to interleukin-10 and IFN-γ, which interact with their receptors in the dimer form[7-8].

Site-specific protein modification technologies are required to link proteins together in a hybrid recombinant-chemical approach[9-12] to make multifunctional proteins. There may be many therapeutic opportunities for multifunctional proteins[2, 13-15]. Proteins linked together to be multivalent (for example, [16]) or dimeric[17-19] have been described using a range of modification technologies. Dimerised proteins may increase avidity while also increasing solution size to prolong circulation times[12, 20]. Recombinant[21] and chemical[22-23] methods have been used to make different cytokine dimers[2].

Disulfide re-bridging conjugation is a site-specific method for protein modification that exploits the reactivity of the two thiols found in a native disulfide (Figure 1A)[24-25]. Mechanistically conjugation occurs by *bis*-alkylation by a sequence of addition-elimination reactions (Figure 1B). Interferon α2 has two disulfides, either of which can be readily conjugated (Figure 2).

FIGURES 1-2

Disulfide re-bridging conjugation has also been used with reagents functionalized at each terminus of a PEG linker (e.g. PEG di(*bis*-sulfone) **4**, Figure 3B) to make bivalent IgG antibody mimetics called Fab-PEG-Fab (FpFs)[26]. FpFs are about the same size in solution as an IgG and can be made to be more stable in the hinge region. FpFs have been shown to be biologically active *in vitro* and *in vivo* with binding affinities comparable to IgGs targeted to the same epitopes[27]. Interestingly, FpFs display a propensity to have slower dissociation rates once bound to their target. Exploiting reduced dissociation rates may be a strategy to increase efficacy by retaining a therapeutic agent for a longer period of time within a target tissue[28-29].

Fc-fusion mimetics known as receptor-PEG-receptor (RpR) have also been prepared[30]. It was found that for an RpR targeted to vascular endothelial growth factor, that the RpR displayed a higher rate of association as well as a slower rate of disassociation. Together, these properties showed that a recombinant-chemical fusion protein mimetic can have higher affinity than a recombinant protein targeting the same epitope.

IFN binds to two cell surface receptors that then dimerise, so much of the solvent accessible surface of IFN is involved in binding[31]. Histidines are generally rare in proteins and contiguous histidines are only generally present in His-tags, which are widely used to simplify protein purification. In an effort to avoid modifying the IFN near a residue that may be involved in binding, IFN was made with an 8-Histidine tag (His_8-tag) at its N-terminus and then site-specifically mono- and di-PEGylated by *bis*-alkylation using the PEG mono-sulfone **3** (Figure 3C)[32]. His-tag conjugation is thought to occur by a similar addition-elimination reaction (Figure 3D) that occurs with the two thiols from a disulfide bond.

Following a recombinant-chemical approach to make IFN-α2 homo-dimers, activity of the IFN homo-dimer was determined. The cytokine was prepared recombinantly with an His_8-tag at its N-terminal (His_8IFN). PEG-di(mono-sulfone) **5** (Figure 3B) was then used to make an IFN homo-dimer by histidine-specific conjugation (His_8IFN-PEG-His_8IFN). His_8IFN was also used to make IFN-PEG-IFN by disulfide re-bridging conjugation using PEG di(*bis*)sulfone **4**. Disulfide conjugation on His_8IFN had not been conducted before this study. Mono-PEGylated and di-PEGylated conjugates were also prepared as controls for comparison. PEG steric shielding should be at a maximum in the diPEGylated species (Figure 2) and the extent of PEG shielding was probed using a low molecular weight PEG (5 kDa) to make mono- and di-PEGylated IFN conjugates by disulfide re-bridging conjugation.

Materials and methods

Preparation of PEG conjugation reagents

PEG *bis*-sulfone reagents **1** were prepared as previously described[25] using commercially available methoxy-terminated PEG mono-amine precursors of 5, 10 and 20 kDa molecular weights. The PEG di-(*bis*-sulfone) reagents were prepared as previously described[26] and further purified by HPLC. Incubation of the PEG *bis*-sulfone precursors (**1** and **4**) for 8 h (37°C) in 50 mM sodium phosphate buffer pH 7.4 containing 150 mM sodium chloride provided PEG mono-sulfone **3** and PEG di-(mono-sulfone) **5** for histidine-specific conjugation.

His_8IFN production

The IFN α-2a (IFN) gene was designed to include eight histidines (His_8) at the N-terminus[32]. The DNA sequence was optimized using Gene Perfect software (Gene Oracle, Mountain View, California) and further optimized by manual removal of rare codons and repetitive sequences to remove double-strand RNA secondary structure. The Shuffle™ T7 Express *E. coli* bacterial strain with inserted plasmids containing the gene for the expression His_8-IFN. After fermentation and lysis, His_8IFN was purified by immobilized metal affinity chromatography (IMAC) followed by fraction pooling and up purification by an anion exchange chromatography (AIEC, HiPrep™ Q FF 16/60 column). Following SDS-PAGE analysis, the fractions containing pure His_8IFN were pooled and the concentration measured by UV absorbance at 280 nm (ε=0.914, 0.9 mg/mL, 70 mL). The concentration was made to 0.5 mg/mL and the solution supplemented with 100 mM sodium chloride, 10% glycerol, protease inhibitor (dilution 1/5000), 1 mM EDTA and 1 mM sodium azide. His_8IFN was then aliquoted into 5 mL cryovials and snap-frozen in liquid nitrogen and stored at -80°C.

SDS-PAGE

SDS-PAGE was conducted using 4-12% Bis-Tris polyacrylamide gels (NuPAGE®; NP0323BOX). Every gel was loaded with Novex® Sharp pre-stained standards (Novex; P/N 57318) and samples were loaded using an appropriately diluted 4×SDS sample buffer (NuPAGE®; NP0007). Gels were run in Xcell SureLock™ gel electrophoresis tanks (Invitrogen; EI0001) and run in MES SDS-PAGE running buffer (×20) from NuPAGE® (NP0002-02). Gels were stained using InstantBlue™ gel stain (Expedeon; ISB01L) and PEG stain (barium chloride and 0.05 M iodide were both from Fisher Scientific (B/0500/53; J/4410/15). Alternatively, silver stain (SilverXpress® Silver staining kit) was used from Invitrogen (LC6100). Densimetric

analysis was conducted using ImageQuant™ LAS 4010 instrument (GE Healthcare, 28-9558-10).

Determination of protein concentration

MicroBCA™ Protein Assay Kit (Thermo Scientific, 23235) containing a solution of albumin standard (2 mg/mL) was diluted in an appropriate buffer (dependent on the sample buffer) to 200 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL and 0 µg/mL (blank). The samples (75-150 µL) were incubated respectively with 75-150 µL MicroBCA™ solution (prepared by mixing solutions A:B:C in the ration of 25:24:1) at 37 ºC for 2 h. The absorbance was then measured (570 nm) to generate a standard curve. For His_8IFN samples, appropriate dilutions (40-60 µg/mL) were made to a concentration within the linear part of the standard curve and the above steps followed. Additionally, for quantification of proteins in small volumes (<900 µL), Nanodrop 2000 spectrophotometer from Thermo Scientific (SPR-700-310L) was used.

Disulfide re-bridging conjugation; preparation of PEG-IFN-PEG, PEG_2-IFN and PEG-IFN

His_8IFN (5 mg; 4.875 mL) in 50 mM sodium phosphate, 20 mM EDTA, pH 7.8 was reduced using DTT (1 M, 15.4 mg, 125 µL) for 30 min at RT, and the DTT was removed by buffer exchange using two pre-equilibrated PD-10 columns. The His_8IFN concentration was quantified using UV at absorbance 280 nm (0.797 mg/mL, 6.5 mL) and was diluted to 0.5 mg/mL (10 mL). PEG_{20} *bis*-sulfone **1** was weighed (5.9 mg, 1.2 eq.) and added to the reduced His_8IFN (250 µL) in a 1:1 ratio (PEG_{20} *bis*-sulfone **1**:IFN). The PEG_{20}-IFN reaction mixture was left for 3 h at RT, then 1 mM glutathione re-oxidising solution (GRS; 50 µL of 50 mM GSH:50 mM GSSG in 50 mM sodium acetate, pH 4.0) was added, and the reaction allowed to proceed for a further 16 h at 20 ºC. SDS-PAGE analysis was conducted on the reaction mixture prior to cation exchange (IEX) purification using a 5 mL HiTrap Macrocap SP column connected to an ÄKTA prime which had been pre-equilibrated with buffer A (100 mM sodium acetate, pH 4.0). The protein conjugates were eluted with a step gradient of buffer B (1.0 M sodium chloride in 100 mM sodium acetate, pH 4.0) where the reaction mixture was loaded manually onto the column (*ca*. 10 mL), and the system was washed for 30 mL, then the gradient was initiated. Fractions containing the desired conjugates were combined and centrifugally concentrated using a VivaSpin column with 10,000 MWCO at 3000×*g*, 4ºC until *ca.* 1.5 mL remained. Once concentrated, the PEG_{20}-IFN and PEG_{20}-IFN-PEG_{20} conjugates were buffer exchanged into 50 mM sodium phosphate containing 20 mM EDTA, pH 7.8. Protein concentration was

estimated by measuring UV absorbance at 280 nm and was confirmed by MicroBCA assay for both PEG_{20}-IFN and $(PEG_{20})_2$-IFN conjugates (330 µg/mL and 147.5 µg/mL, respectively). The yield for both PEG_{20}-IFN and PEG_{20}-IFN-PEG_{20} conjugates was 28.8% and 12%, respectively. PEG_{10}-IFN and PEG_{10}-IFN-PEG_{10} were prepared similarly in yields of 14% and 16%, respectively. PEG_5-IFN and PEG_5-IFN-PEG_5 were also prepared with yields of 29% and 34.5%, respectively.

Preparation of PEG_{20}-His_8IFN and $(PEG_{20})_2$-His_8IFN

PEG_{20} *mono*-sulfone **3** (0.502 mL, 2.5 eq.) was added to the His_8IFN solution (2.837 mL at 1.41 mg/mL) and incubated at 20ºC for 16 h. The reaction mixture was cooled to 4ºC and then incubated with sodium triacetoxyborohydride (50 mM) dissolved in DMSO at 4ºC for 1.5 h. The crude mixture (2×2.5 mL) was buffer exchanged into 100 mM sodium acetate, pH 4.0, using a pre-equilibrated PD-10 desalting column. The reaction mixture was loaded into a 5 mL HiTrap Macrocap SP cation exchange column (CIEC, GE Healthcare; 28-9508-59) using an ÄKTA prime plus fitted with a 5 mL loop which had been pre-equilibrated with buffer A (100 mM sodium acetate, pH 4.0). The His_8IFN species were eluted using a step gradient of buffer B (1.0 M sodium chloride in 100 mM sodium acetate, pH 4.0) with the following steps of 27%, 36%, 55% and 100%, with each step eluting over six column volumes. Fractions (2 mL) collected over the step gradient were then analyzed by SDS-PAGE and the desired conjugates were combined and concentrated using a VivaSpin column with 10,000 MWCO at 3000×*g*, 4ºC, until the volume was approximately 1.5 mL. The PEG_{20}-His_8IFN and $(PEG_{20})_2$-His_8IFN conjugates were buffer exchanged into buffer A to decrease the salt concentration and enable the conjugates to bind to the column for a second CIEC purification cycle. Protein concentration and yield was determined by measuring UV absorbance at 280 nm for both PEG_{20}-His_8IFN and $(PEG_{20})_2$-His_8IFN conjugates 1.054 mg (26%) and 0.483 mg (12%), respectively. UV absorbance at 280 nm and MicroBCA™ assay were conducted to quantify protein concentration after the second purification cycle and the final yields of PEG_{20}-His_8IFN and $(PEG_{20})_2$-His_8IFN conjugates were 0.7 mg (17.6%) and 0.29 mg (7.13%), respectively.

Preparation of IFN dimer, His_8IFN-PEG_{20}-His_8IFN by HIS-tag conjugation

PEG_{20} di(*mono*)sulfone **5** (0.515 mL,1 eq.) was added to His_8IFN solution (0.5 mL at 3 mg/mL) and incubated for 16 h at 20ºC. The reaction mixture was cooled to 4ºC and then incubated with sodium triacetoxyborohydride (100 mM) dissolved in DMSO at 4ºC for 1.5 h. The crude mixture was then buffer exchanged into 100 mM sodium acetate, pH 4.0, using a pre-equilibrated PD-10 desalting column. The reaction

mixture was then loaded into a 5 mL HiTrap Macrocap SP CIEC column using an ÄKTA prime plus fitted with a 5 mL loop which had been pre-equilibrated with buffer A (100 mM sodium acetate, pH 4.0). A buffer B (1 M sodium chloride, 100 mM sodium acetate, pH 4.0) step gradient of 27%, 36%, 55%, 80% and 100% was used to elute the different His_8IFN species. Fractions containing His_8IFN-PEG_{20}-His_8IFN were combined and concentrated using a VivaSpin column with 10,000 MWCO at 3000×*g*, 4ºC, until the volume was approximately 1.5 mL. The conjugate solution was buffer exchanged into 50 mM sodium phosphate containing 150 mM sodium chloride, pH 7.4 and the protein concentration and yield was measured by UV at 280 nm for His_8IFN-PEG_{20}-His_8IFN to be 0.407 mg/mL (13.5%) yield. SEC was used to separate His_8IFN-PEG_{20}-His_8IFN from trace amount of unmodified His_8IFN. The mixture was loaded into a 120 mL Superdex™ 200 prep grade column 16/60 using an ÄKTA prime plus fitted with a 2 mL loop which had been pre-equilibrated with 50 mM sodium phosphate containing 150 mM sodium chloride, pH 7.4. Collected fractions were analyzed by SDS-PAGE and visualized with silver stain, and the purest fractions of His_8IFN-PEG_{20}-His_8IFN were combined and concentrated using pre-equilibrated VivaSpin column with 10,000 MWCO at 3000×*g*, 4ºC, until approximately 1.5 mL remained. The His_8IFN-PEG_{20}-His_8IFN concentration and yield (0.030 mg/mL, 1.5%) was measured by UV at 280 nm and MicroBCA assay.

Preparation of IFN dimer, IFN-PEG-IFN by disulfide re-bridging conjugation

His_8IFN (2 mg/mL, 5 mL) in 50 mM sodium phosphate 20 mM EDTA, pH 7.8, was reduced using DTT (1 M, 15.4 mg, 125 µL) for 30 min at RT, and the DTT was removed by buffer exchange using three pre-equilibrated PD-10 columns. Protein concentration was quantified by UV at 280 nm (0.752 mg/mL, 11 mL) and His_8IFN was diluted to 0.5 mg/mL (15 mL), where one half (7.5 mL, 4.1 mg) of this reduced His_8IFN was used to prepare IFN-PEG_{10}-IFN and the other half used to prepare IFN-PEG_{20}-IFN. For IFN-PEG_{20}-IFN, PEG_{20} di(*bis*)sulfone **4** (2.5 mg) was added to the solution of reduced His_8IFN (106 µL) in a 2:1 ratio (PEG_{20} di(*bis*)sulfone **4**:IFN). The reaction mixture was left for 5 h at 20ºC, then GRS (1 mM, 153.1 µL of 50 mM GSH:50 mM GSSG in 50 mM sodium acetate, pH 4.0) was added, and the reaction was allowed to proceed for a further 16 h at 20ºC. SDS-PAGE analysis was conducted on the reaction mixture prior to and after GRS addition. The IFN-PEG_{20}-IFN conjugate was then incubated with sodium triacetoxyborohydride (100 mM dissolved in DMSO) at 4ºC for 1.5 h. The reaction mixture (7.5 mL) was then buffer exchanged into 100 mM sodium acetate, pH 4.0, using three pre-equilibrated PD-10 desalting columns. CIEC purification was conducted with a 5 mL Macrocap SP ion

exchange column using an ÄKTA Prime Plus fitted with two 5 mL loops which had been pre-equilibrated with buffer A (100 mM sodium acetate, pH 4.0). The protein conjugates were eluted with a step gradient of buffer B: 1.0 M sodium chloride in 100 mM sodium acetate, pH 4.0, of 45%, 55% and 100%.

IFN-PEG_{10}-IFN and His_8IFN mixtures were separately combined and concentrated using a VivaSpin column with 10,000 MWCO at 3000×*g*, 4ºC until approximately 1.5 mL remained. The mixtures were buffer exchanged using pre-equilibrated NAP-10 columns into 50 mM sodium phosphate containing 150 mM sodium chloride, pH 7.8. Protein concentration and yield was calculated by measuring UV at 280 nm for both IFN-PEG_{20}-IFN + His_8IFN, IFN-PEG_{10}-IFN + His_8IFN mixtures (0.682 mg/mL, 27.3% and 1.12 mg/mL, 27.3%, respectively).

SEC purification of IFN-PEG_{20}-IFN + His_8IFN and IFN-PEG_{10}-IFN + His_8IFN mixtures was performed to separate His_8IFN from IFN-PEG_{20}-IFN and IFN-PEG_{10}-IFN. The mixtures were loaded separately onto the Superdex 200 Prep Grade Column 16/60 120 mL column, which had been pre-equilibrated with 50 mM sodium phosphate containing 20 mM EDTA, pH 7.8, using an ÄKTA Prime Plus fitted with a 2 mL loop. The protein conjugates were eluted over 120 mL and the fractions collected were then analyzed by SDS-PAGE and stained using silver stain. Fractions were combined and concentrated using a VivaSpin column with 10,000 MWCO at 3000×*g*, 4ºC until approximately 1.5 mL remained. Protein concentration for both IFN-PEG_{20}-IFN and IFN-PEG_{10}-IFN were measured by UV at 280 nm and confirmed by MicroBCA assay to 0.035 and 0.046 mg/mL, respectively. Final yields for IFN-PEG_{20}-IFN and IFN-PEG_{10}-IFN conjugates were 1.4% and 1.12%, respectively.

Assessment of conjugate stability

Samples were incubated at 4ºC for 7 days and monitored by SDS-PAGE with the resulting gels stained by InstantBlue™ and PEG stain. For conjugated proteins with a low concentration, silver stain was used to detect the conjugate and any free protein. Accelerated stability assessments were conducted by first buffer exchanging samples into 10 mM ammonium bicarbonate, pH 8.0, and protein concentrations were diluted to 50 µg/mL, and then 27 µL was transferred into 4×0.5 mL sample vials. For each protein product, two of the sample vials were made up to 30 µL with the addition of 3 µL of 10 mM ammonium bicarbonate, pH 8.0, while the two remaining sample vials are made up to 30 µL with 100 mM DTT solution. For each protein product, two sample vials (one with DTT and one without) were heated on a metal heating block to 90ºC for 10 min. The two remaining sample vials (one with DTT and one without) were then heated in the metal heating block for 1 h at 50ºC.

Once all sample vials for each protein product were cooled to RT, 22 µL was removed from each sample vial and transferred into new labeled sample vials containing loading dye (11 µL). The sample/loading dye mixtures were subsequently analyzed by SDS-PAGE and stained with InstantBlue™ and PEG stain, to identify if the protein conjugates underwent de-conjugation as determined by the presence of unmodified His_8IFN.

Antiviral potency assay

The human lung fibroblast A549 cell line (HPACC; 86012804) was maintained in Dulbecco's Modified Eagles medium (DMEM) (Gibco/Fisher Scientific; VX21969035) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco/Fisher Scientific; VX25030024), 50 units/mL penicillin (PAA; P11-101), 2 mM L-glutamine (Sigma; G7513-100 mL) and 50 µg/mL streptomycin. A549 cells were infected using encephalomyocarditis virus (EMCV) (ATCC; VR-129B). Sample controls were NIBSC IFN standard (HPACC; 95/650) and Pegasys® (Roche). Phosphate buffered saline (PBS), pH 7.4 (BPE399-20); formaldehyde (40%) (F/145/PB17) and SDS (10%) (BPE 2436-1) were supplied from Fisher Scientific. Methyl violet 2B was from Sigma Aldrich (198099). Microplate reader was supplied from Dynex Technologies/Opsys MR and the microplate shaker was supplied from VWR (444-7094). To conduct the antiviral assay, A549 cells were plated (1.7×10^5 cells/mL at 50 µL/well) in 96 well flat-bottomed tissue culture plates, then incubated at 37ºC, 5% CO_2, overnight until the cells were 80% confluent. Serial dilutions (2-fold) of the IFN samples were prepared in DMEM/10% FBS in 96 well V-bottom plates and diluted samples (50 µL) were transferred to flat-bottom plates and incubated for 24 h at 37ºC, 5% CO_2. Protein samples were tested in triplicate and control wells contained only cells (negative control) or cells with virus (positive control). After incubation, the protein sample and media were discarded and EMCV (50 µL), pre-titrated to achieve complete killing in 24 h in control wells, was added to all wells except for the negative control wells where, 50 µL/well of DMEM/2% FBS was added. The plate was then incubated for 21 h at 37ºC after which *ca.* 80% cell death occurred in the control wells. Media was then discarded and the wells were washed with 300 µL/well of PBS added. Plates were then incubated for 30 min at RT with 50 µL/well of 4% formaldehyde/0.5% methyl violet. After incubation, the plates were washed twice with PBS (200 µL/well) and tap-dried. Dye was solubilized by 20 min agitation in 50 µL/well of 2% SDS and the absorbance measured at 570 nm.

The interferon concentration (the log reciprocal of the interferon dilution) was plotted against the absorbance achieved and the reduction in cell viability was fitted

to a sigmoidal dose-response curve. Data was only considered valid under two criteria, i) the variation between the straight part of the sigmoidal curve and the data points was small enough to be accurately represented by a straight line (absolute sum of squares (r value)) and ii) the ED_{50} for the internal control, NIBSC IFN α, was within the expected range. If the data achieved was not in line with this criterion, the data were not used. Accepted potency data was then turned into international units (IU) to allow for direct comparisons to be made with data described in the literature. GraphPad Prism 5 was used for all data analysis.

Antiproliferative assay

The human Negroid Burkitt's lymphoma Daudi cell line (HPACC, 85011437) was maintained in RPMI 1640 media supplemented with 10% FBS, 50 units/mL penicillin and 50 µg/mL streptomycin. Daudi cells were plated (1.7×10^5 cells/mL at 100 µL/well) in a 96 well round-bottomed tissue culture plates and these were incubated at 37ºC, 5% CO_2. Serial dilutions (three-fold) of IFN α controls (NIBSC IFN and Pegasys®) were conducted in 1.5 mL centrifuge tubes using RPMI 1640/10% FBS. IFN controls were pipetted into the plate (100 µL/well) from low to high dilutions in duplicate, with each well having a final volume of 200 µL/well. Control wells contained only media without cells (negative control) or cells with media (as positive control). The plates were incubated for 72 h at 37ºC, 5% CO_2. Methyl thiazolyl tetrazolium (MTT, 5 mg/mL, Sigma) was dissolved in PBS and was prepared 10 min before addition to the wells. MTT solution was filtered through a 0.2 µm syringe filter and then added (20 µL) to each well and the plates incubated for 3 h at 37ºC, 5% CO_2. Plates were then centrifuged at 1500×*g* for 10 min, allowing formazan crystals to sediment. Using a multichannel pipette, the supernatant was carefully removed and 100 µL/well non-sterile DMSO was added to solubilize the crystals by agitation. Absorbance was then measured at 570 nm. Cell viability (%) was plotted against control wells using site-specific binding with Hill slope on a semi-log graph (GraphPad Prism 5). The interferon concentration (the log reciprocal of the interferon dilution) was plotted against the absorbance achieved and the reduction in cell viability was fitted to a sigmoidal dose-response curve. Data was only accepted if the variation between the straight part of the sigmoidal curve and the data points was small enough to be accurately represented by a straight line (absolute sum of squares (r value)).

Results and Discussion

Reagent synthesis.

Terminally functionalized PEG di(*bis*-sulfones) **4** (Figure 3B) capable of *bis*-alkylation conjugation at each PEG terminus were prepared using commercially available 10 and 20 kDa PEG diamines (NH_2-PEG-NH_2)[26] and the precursor *bis*-sulfone linker[25]. Likewise the, PEG *bis*-sulfones **1** were prepared from commercially available 5, 10 and 20 kDa PEG amine[25]. The *mono*-sulfone adducts (**3** and **5**) were obtained by elimination of toluene sulfinic acid **2**[24].

FIGURE 3

Preparation of His_8IFN

Recombinant IFN α-2a fused with an eight-histidine tag (His_8) was prepared by using the SHuffle™ T7 expression system which can correctly produce disulfide containing proteins in high yields within the cytoplasm of *E. coli*[33]. The His_8-tag was attached between the cysteine and methionine at the N-terminus end of IFN[32]. Lysis was conducted firstly by sonication and then cross flow-filtration to remove the soluble His_8IFN from the cell debris. As His_8IFN was expressed as a soluble protein, purification was conducted at physiological pH to maintain the stability and activity of the protein. His_8IFN was purified in two steps, first using immobilized metal affinity chromatography (IMAC) taking advantage of the presence of the His_8-tag. His_8IFN was then eluted over an anion exchange column (AIEC).

The identity of pure His_8IFN (Figure 4A) was confirmed by Western blot (Figure 4B). His_8IFN was prepared two times in 500 mL cultures with a final yield after purification of between 60-70 mg. His_8IFN was stored in small vials at -80ºC (0.5 mg/mL) for conjugation reactions. The biological potency of His_8IFN was assessed *in vitro* by the prevention of infection of A549 cells by EMCV[34]. The His_8IFN displayed a specific activity of 231 ± 10.95 MIU/mg, whilst the positive control of NIBSC (non-his-tagged) IFN α-2a displayed an activity of 254 MIU/mg. In literature His_8IFN has been reported to display an ED_{50} of 7 pg/mL[32]. The activity of non-his-tagged interferon α2 is reported to be 1.4×10^8 IU/mg.

FIGURE 4

Preparation of the His_8IFN-PEG_{20}-His_8IFN homo-dimer by histidine-specific conjugation

The IFN-PEG-IFN homo-dimer (His_8IFN-PEG_{20}-His_8IFN) derived from histidine-specific conjugation was achieved using PEG_{20} di-(*mono*-sulfone) **5** (1 eq. in 200 mM sodium phosphate containing 150 mM sodium chloride) and three molar equivalents

of His_8IFN (3.3 mg/mL, 0.5 mL in 50 mM sodium acetate, pH 5.3, containing 35 μM hydroquinone). Histidine-specific conjugation generally requires a slightly acidic pH to maintain site-selectivity, so use of the *mono*-sulfone reagents (**3** and **5**) is one way to better target the His_8-tag on the IFN. The reaction mixture was incubated for 16 h at 20ºC at approximately pH 6.5 and then treated with 100 mM sodium tri-acetoxy-borohydride (STAB) to reduce the electron-withdrawing carbonyl to prevent retro-Michael reactions. A band at 70-80 kDa in the SDS-PAGE was consistent for the formation of His_8IFN-PEG_{20}-His_8IFN. The molecular weight of each IFN is *ca.* 20 kDa (2×20 kDa = 40 kDa) and PEG often migrates in SDS-PAGE to about twice the molecular weight of the protein standards.

However, SDS-PAGE analysis showed that conversion was low with a significant amount of unconjugated IFN still present in the reaction mixture. Since the goal was to isolate the IFN homo-dimer, the reaction mixture was subjected to purification by CIEX. The His_8IFN-PEG_{20}-His_8IFN dimer was separated from the unconjugated PEG species (Figure 5A, lane 3), but the desired His_8IFN-PEG_{20}-His_8IFN dimer could not be separated from other high molecular weight IFN impurities even after a second purification by CIEX (Figure 5A, lane 4).

FIGURE 5

The conjugation was repeated and a single CIEX purification was conducted followed by purification of the fractions containing His_8IFN-PEG_{20}-His_8IFN by SEC to remove the high molecular weight species (Figure 5A, lane 5). An anti-IFN Western blot was conducted to confirm the presence of IFN (Figure 5A, lane 6) in the purified His_8IFN-PEG_{20}-His_8IFN.

The final yield of His_8IFN-PEG_{20}-His_8IFN homo-dimer after one cycle of IEX followed by one cycle of SEC purification was 1.5% (Table 1) based on UV and MicroBCA™ assays. PEG_{20}-His_8IFN and $(PEG_{20})_2$-His_8IFN were prepared using the PEG *mono*-sulfone **3**. Good purification of these modified proteins was accomplished using two IEX steps and the purified conjugates were isolated in 17.6% (0.7 mg) and 7.1 % (0.29 mg) yields, respectively (Figure 5B, Lanes 3-4, Table 1).

TABLE 1

The purified His_8IFN-PEG_{20}-His_8IFN was stored at 4°C for 2 months (50 mM sodium phosphate containing 150 mM sodium chloride, pH 7.8). No free His_8IFN was observed in the SDS PAGE using silver stain, indicating there was no de-conjugation. Conjugate stability for His_8IFN-PEG_{20}-His_8IFN was also evaluated at 50ºC and 90°C in the presence and absence of DTT (Figure 5C). No free His_8IFN was observed. The imidazole side chain of histidine is a weaker nucleophile than free

thiol. Since conjugate formation occurs via a reaction mechanism that involves a sequence of equilibria (Figure 3D), it was important to ensure that bonds formed between the His_8-tag and the PEG di-(*mono*-sulfone) **5** had been stabilized by the treatment with STAB to reduce the electron-withdrawing carbonyl in the reagent after conjugation. Both PEG_{20}-His_8IFN and $(PEG_{20})_2$-His_8IFN conjugates that were also prepared in this study were evaluated at 50ºC and 90°C in the presence and absence of DTT (Figure 5D). No de-conjugation was observed for either of these conjugates.

Preparation of IFN-PEG-IFN homo-dimers by disulfide re-bridging conjugation

Conjugation of the disulfide thiols has not previously been conducted on His_8IFN. Once the IFN thiols are liberated after disulfide reduction, thiol addition to the PEG *bis*-sulfone reagents (**1** and **4**) is much more favored compared to the addition reaction of the imidazole side-chain of histidine. IFN has two accessible disulfides and IFN maintains its tertiary structure in the presence of DTT after reduction of the disulfides[35].

Bis-alkylation conjugation of His_8IFN was first conducted using PEG *bis*-sulfone **1** (5, 10 and 20 kDa) to prepare the corresponding PEG-IFN and PEG_2-IFN conjugates (Figure 6A, lanes 3-8, Table 1). These conjugates were isolated from the same reaction mixture using 1 molar equivalent of PEG *bis*-sulfone **1** to protein, and the PEG_2-IFN conjugate was easily isolated as a co-product. The conjugation conversion and the purification process comprising a single CIEX for the His_8IFN-derived conjugates is similar to what has been observed with IFN[36] that does not possess a His-tag.

FIGURE 6

The IFN-PEG-IFN homo-dimers were prepared using a 2:1 molar ratio of His_8IFN to PEG di(*bis*)sulfone **4** (10 and 20 kDa reagents). His_8IFN was reduced using 20 mM DTT and then the protein was buffer exchanged into 50 mM sodium phosphate 20 mM EDTA. A pH of 7.8 was used because the PEG di(*bis*)sulfone **4** readily undergoes elimination of the toluene sulfinic acid **2** (Figure 3B) to generate the PEG di-(*mono*-sulfone) **5** *in situ* followed by the Michael reaction of free protein thiols. Reduced His_8IFN (0.5 mg/mL, 7.5 mL) was allowed to incubate for 5 h at 20ºC separately with the 10 kDa and 20 kDa PEG di(*bis*-sulfone)s **4**. To re-oxidise the unconjugated thiols of the remaining disulfide, a glutathione re-oxidising solution (GRS) was added (50 mM, 153 µL) and the reaction mixture was allowed to incubate a further 16 h at 20ºC before being treated with STAB to reduce the electron-withdrawing carbonyl to prevent retro-Michael reactions.

Purification of the two IFN-PEG-IFN homo-dimers was accomplished by CIEX followed SEC (Figure 6A, lanes 9 and 10). SEC was required to purify the desired IFN homo-dimer from the starting His_8IFN which could not be separated by CIEX. Anti-IFN Western blots substantiated the presence of IFN in the homo-dimers while, importantly, confirming the absence of the starting His_8IFN in the purified products.

UV and BCA quantification was conducted and the yields obtained for the purified IFN-PEG_{20}-IFN and IFN-PEG_{10}-IFN were 0.85% (35 µg/mL) and 1.1% (46 µg/mL), respectively. As with the IFN homo-dimer derived from histidine-specific conjugation (His_8IFN-PEG_{20}-His_8IFN), the isolated yields for the purified IFN homo-dimers were low. Clearly the SEC purification step is not optimal for a purification process, since SEC can result in reduced yields for small scale reactions. The reaction was repeated to make both the 10 and 20 kDa IFN homo-dimers, but the yields were not improved.

Storage of the disulfide re-bridged conjugates in 50 mM sodium phosphate, 150 mM NaCl, pH 7.8, for 7 days at 4ºC did not show any evidence of de-conjugation (Figure 6B). Accelerated stability studies were also performed by incubating the IFN homo-dimers and their mono-PEGylated variants (*ca.* 40 µg/mL) at 90ºC (10 min) with or without 20 mM DTT (Figure 6C-D). No free His_8IFN was observed indicating that there was no de-conjugation of the IFN from the PEG-linker.

In vitro potency of IFN homodimers

IFN is a pleiotropic cytokine that has numerous distinct biological properties; one of these is antiviral activity. This can be assessed *in vitro*, by the prevention of infection of A549 cells by EMCV[34]. The relative activity of the IFN homo-dimers and PEG-IFN conjugates was determined by comparing the dose (concentration) of the sample, which displays 50% prevention of infection in cells or (50% effective dose or ED_{50}) *in vitro* to the dose of the National Institute for Biological Standards and Control (NIBSC) reference standard IFN α-2a. The ED_{50} was then used to calculate the specific activity of each His_8IFN conjugate. In addition to the NIBSC IFN control, Pegasys® was also used. Pegasys ® is a 40 kDa branched PEG-IFN α-2a conjugate that is a marketed product for the treatment of hepatitis C.

The IFN homo-dimers retained activity that was comparable or better than Pegasys ® (Figure 7A-B and Table1). Both PEG_{20}-derived homo-dimers (His_8IFN-PEG_{20}-His_8IFN and IFN-PEG_{20}-IFN) displayed less activity than the corresponding mono-PEGylated species, but more activity than the corresponding di-PEGylated species. However, the IFN homo-dimer IFN-PEG_{10}-IFN did retain more activity than

the corresponding PEG_{10}-IFN mono-PEGylated conjugate (Figure 7B, Table 1), suggesting there could be a dependency on PEG size linking the two IFNs.

FIGURE 7

The histidine-specific mono-PEGylated conjugate (PEG_{20}-His_8IFN) displayed greater retained activity compared to the disulfide re-bridged mono-PEGylated conjugates, PEG_{10}-IFN and PEG_{20}-IFN (Table 1). This is consistent with a previous study for the PEGylating IFN at the N-terminal His-tag[32]. When comparing the two PEG_{20}-derived homo-dimers, the histidine-specific homo-dimer (His_8IFN-PEG_{20}-His_8IFN) also displayed more activity than the IFN-PEG_{20}-IFN dimer.

Due to the site specificity of the disulfide re-bridging PEGylation, the activity of PEG_{10}-IFN and PEG_{20}-IFN were comparable as previously reported[24]. Interestingly, the PEG_5-IFN conjugate displayed considerably more activity than any of the higher molecular weight PEG-IFN conjugates (Figure 7 and Table 1), suggesting that when PEG_5 is site-specifically conjugated by disulfide re-bridging conjugation, this molecular weight of PEG is below a steric shielding threshold at these sites of conjugation.

An antiproliferative assay using Daudi cells was conducted, but due to the large quantity of sample required, only the disulfide re-bridged mono-PEGylated and IFN homo-dimers were evaluated. As with the antiviral assay, the PEG_{10} homo-dimer (IFN-PEG_{10}-IFN, 1.52 ± 0.47 MIU/mg) again displayed higher activity than the PEG_{20} homo-dimer (IFN-PEG_{20}-IFN, 0.81 ± 0.09 MIU/mg). The activity of the starting His_8IFN in this assay was 329.36 ± 87.98 MIU/mg.

Conclusion

Interferon (IFN) homo-dimers were prepared by site-specific *bis*-alkylation conjugation of IFN. PEG (10 and 20 kDa) di-functionalized linkers were used to conjugate two IFN molecules to the two PEG termini. Both histidine-specific and disulfide re-bridging conjugation processes were examined to isolate the purified the IFN homo-dimers. The His_8IFN-PEG_{20}-His_8IFN obtained by histidine-specific conjugation displayed marginally greater *in vitro* antiviral activity than the IFN-PEG_{20}-IFN homo-dimer obtained by disulfide re-bridging conjugation. This result is consistent with previous observations in which enhanced retention of activity was made possible by conjugation to an N-terminal His-tag on the IFN. Comparison of the antiviral and antiproliferative activities of the two IFN homo-dimers prepared by disulfide re-bridging conjugation indicated that IFN-PEG_{10}-IFN was more biologically

active than IFN-PEG_{20}-IFN. This result suggests that the size of PEG may influence the antiviral activity of IFN-PEG-IFN homo-dimers.

Acknowledgements.

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Figures

Figure 1

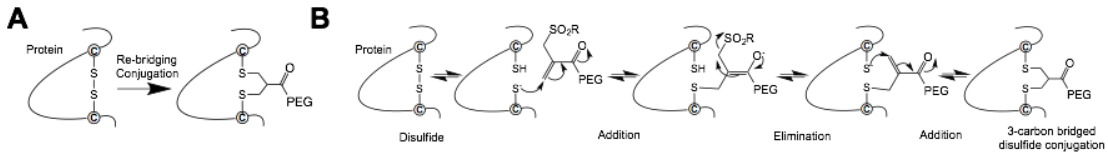

Figure 1. (A) Disulfide re-bridging conjugation is a site-specific strategy for modifying proteins by *bis*-alkylation where a native disulfide is linked with a three-carbon bridge that is bound to PEG. **(B)** *Bis*-alkylation conjugation occurs by a sequence of addition and elimination steps resulting in the formation of a three-carbon bridge.

Figure 2

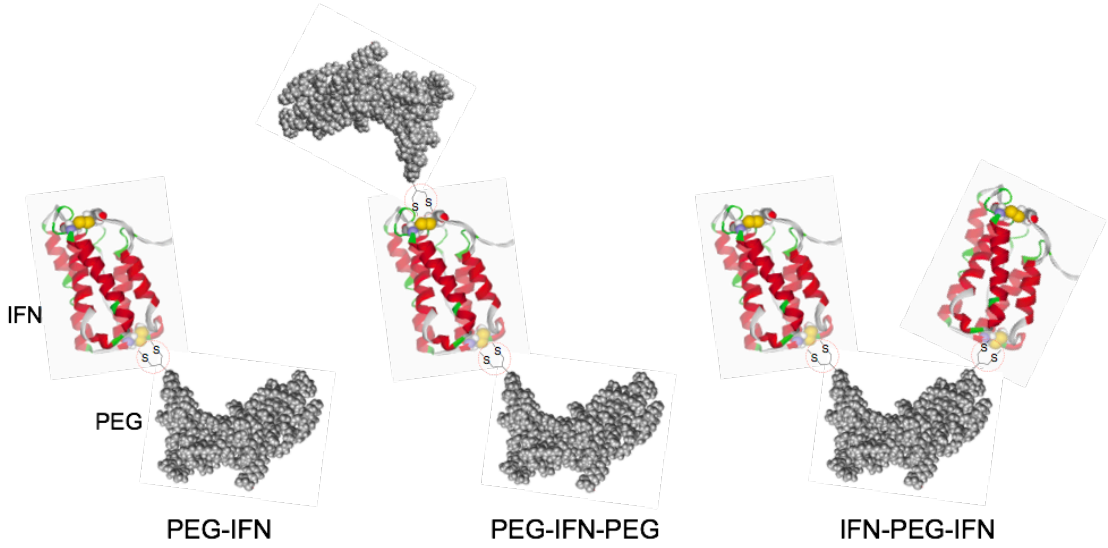

Figure 2. The range of interferon-α2 (IFN) conjugates that can be accomplished by disulfide re-bridging conjugation at either of the two disulfides in IFN (Cys1-98 or Cys29-Cys138). Reagents that are functionalized at each PEG terminus can be used to prepare IFN homo-dimers (IFN-PEG-IFN). IFN can also be prepared with a His-tag (His_8 in this study) at the N-terminus which can be site-specifically conjugated with the same reagents used for disulfide re-bridging conjugation.

Figure 3

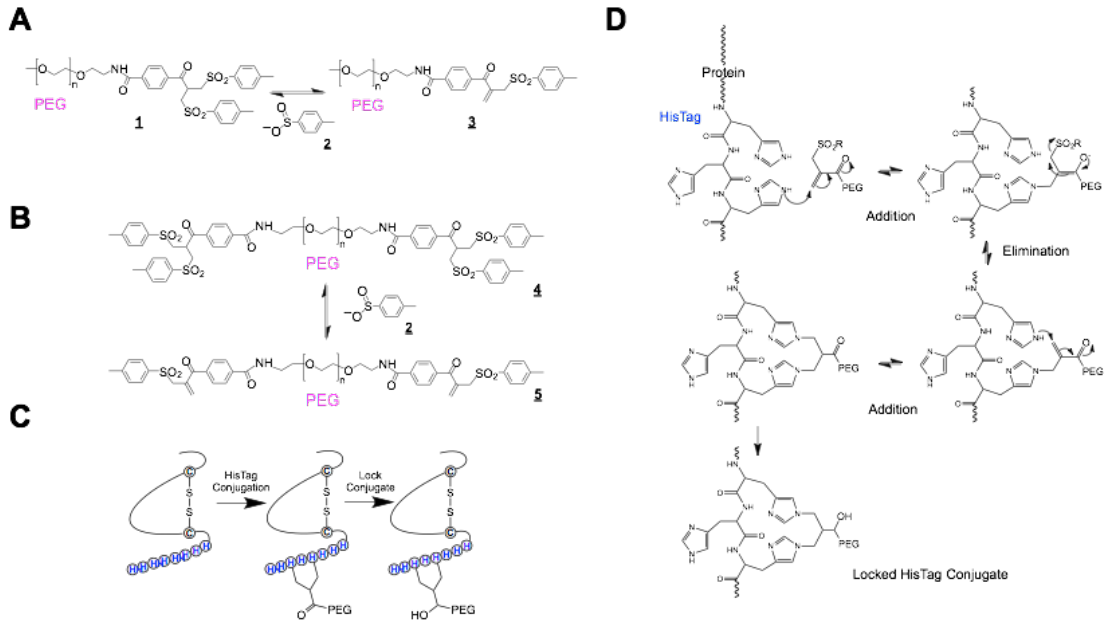

Figure 3. (A) PEG *bis*-sulfone **1** undergoes elimination of toluene sulfinic acid **2** leaving group to generate the PEG *mono*-sulfone **3** that undergoes *bis*-alkylation conjugation with a protein. **(B)** PEG linkers can be functionalized at each terminus to give the corresponding PEG di(*bis*-sulfone) **4** which can undergo elimination to generate the PEG di(*mono*-sulfone) **5** that can undergo site-specific conjugation to two proteins. **(C)** A protein His-tag can also be a target for *bis*-alkylation conjugation. **(D)** *Bis*-alkylation conjugation to a His-tag occurs by a similar set of addition and elimination reactions, which occur for two thiols in a reduced disulfide. After conjugation, the electron-withdrawing group can be reduced to an alcohol to lock the His-tag conjugate.

Figure 4

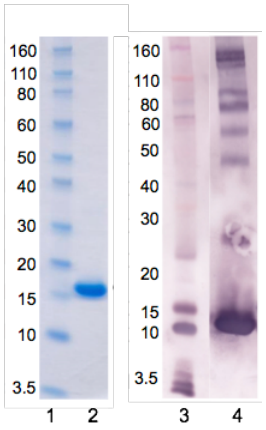

Figure 4. Characterisation of the final His_8IFN product by **(A)** SDS-PAGE (n=2) (InstantBlue™ stain), *Lane 1:* Novex pre-stained markers and *Lane 2:* purified His_8IFN. **(B)** Anti-IFN Western blot (n=1), *Lane 3:* protein markers and *Lane 4:* His_8IFN.

Figure 5

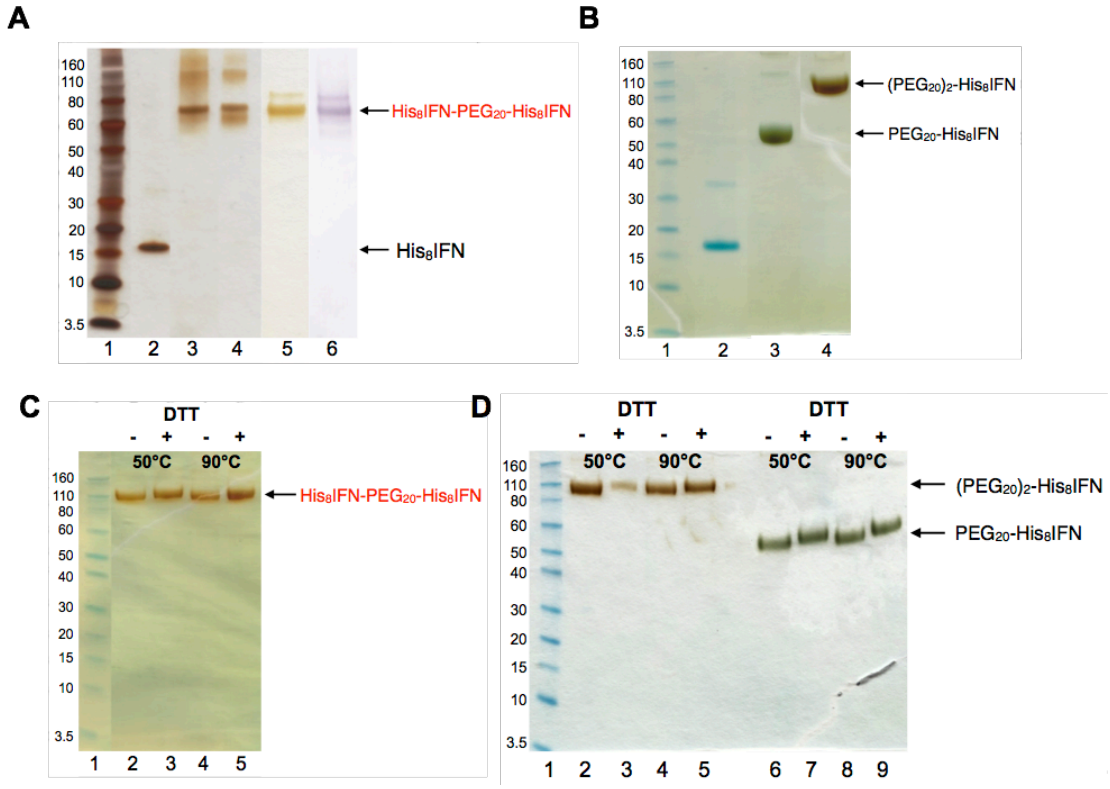

Figure 5. (A) SDS-PAGE (silver stain) of CIEX purified His_8IFN-PEG_{20}-His_8IFN. *Lane 1:* Novex pre-stained markers, *Lane 2:* His_8IFN α-2a, *Lane 3:* one pass CIEX purified His_8IFN-PEG_{20}-His_8IFN, *Lane 4:* two-step CIEX purified His_8IFN-PEG_{20}-His_8IFN. *Lane 5:* His_8IFN-PEG_{20}-His_8IFN after one CIEX and one SEC step and Lane 6: Anti-IFN Western blot of His_8IFN-PEG_{20}-His_8IFN. **(B)** SDS-PAGE (InstantBlue™ and PEG stain) of final PEG_{20}-His_8IFN and $(PEG_{20})_2$-His_8IFN, *Lane 1*: Novex pre-stained markers, *Lane 2:* His_8IFN, *Lane 3:* PEG_{20}-His_8IFN, *Lane 4:* $(PEG_{20})_2$-His_8IFN. **(C)** SDS-PAGE (InstantBlue™ and PEG stain) analysis of His_8IFN-PEG_{20}-His_8IFN homo-dimer incubated ± DTT at 50ºC and 90ºC, *Lane 1:* Novex pre-stained markers, *Lane 2:* -DTT 50ºC, *Lane 3:* +DTT 50ºC, *Lane 4:* -DTT 90ºC, *Lane 5:* +DTT 90ºC. **(D)** SDS-PAGE (InstantBlue™ and PEG stain) analysis of PEG_{20}-His_8IFN (Lanes 6-9) and $(PEG_{20})_2$-His_8IFN (Lanes 2-5) stressed with ± DTT at 50ºC and 90ºC. His_8IFN-PEG_{20}-His_8IFN, PEG_{20}-His_8IFN and $(PEG_{20})_2$-His_8IFN were found to stable to de-conjugation.

Figure 6

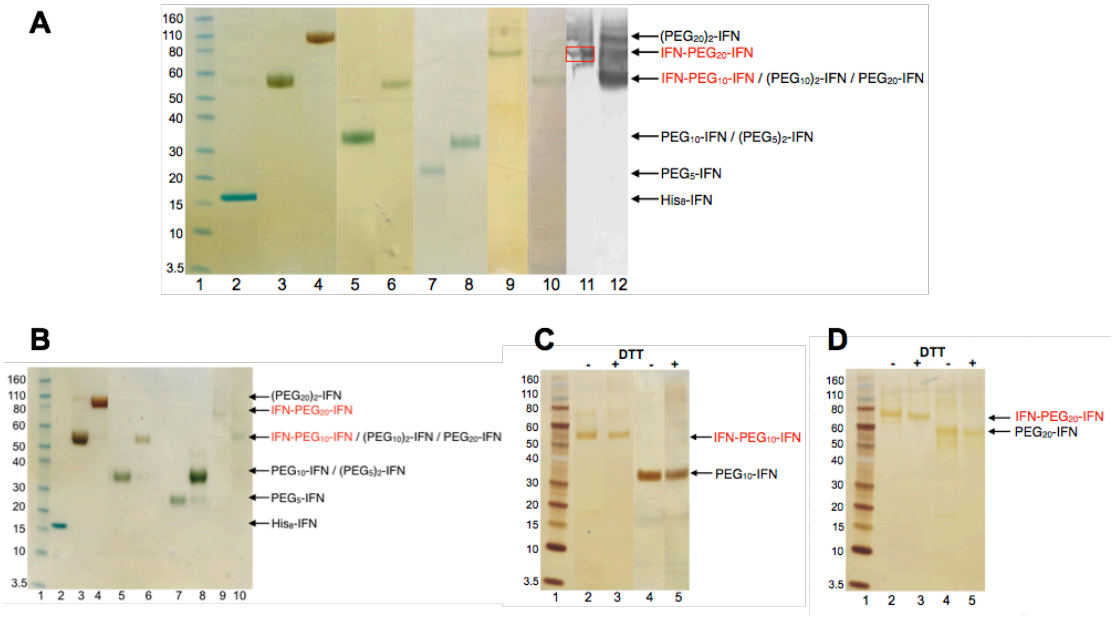

Figure 6. **(A)** SDS-PAGE analysis of the conjugates prepared by disulfide re-bridging conjugation of His_8IFN (InstantBlue™ and PEG stain) *Lane 1:* Novex pre-stained markers, *Lane 2:* His_8IFN, *Lane 3:* PEG_{20}-IFN, *Lane 4:* $(PEG_{20})_2$-IFN, *Lane 5*: PEG_{10}-IFN, *Lane 6:* $(PEG_{10})_2$-IFN, *Lane 7:* PEG_5-IFN, *Lane 8:* $(PEG_5)_2$-IFN. *Lane 9:* IFN-PEG_{20}-IFN, *Lane 10:* IFN-PEG_{10}-IFN, *Lanes 11 and 12:* Anti-IFN Western blot of IFN-PEG_{20}-IFN and IFN-PEG_{10}-IFN. **(B)** Thiol conjugated IFN conjugates incubated at 4ºC for 7 days. *Lane 1:* Novex pre-stained markers, *Lane 2:* His_8IFN, *Lane 3:* PEG_{20}-IFN, *Lane 4:* $(PEG_{20})_2$-IFN, *Lane 5:* PEG_{10}-IFN, *Lane 6:* $(PEG_{10})_2$-IFN, *Lane 7:* PEG_5-IFN, *Lane 8:* $(PEG_5)_2$-IFN, *Lane 9:* IFN-PEG_{20}-IFN, *Lane 10:* IFN-PEG_{10}-IFN. No evidence of free or aggregated protein was observed by SDS-PAGE analysis using InstantBlue™ and PEG stain. **(C and D)** SDS-PAGE (silver stain) analysis of disulfide-conjugated IFN conjugates stressed with ± DTT for 10 min at 90ºC. **(C)** *Lane 1:* Novex pre-stained markers, *Lane 2:* IFN-PEG_{10}-IFN -DTT, *Lane 3:* IFN-PEG_{10}-IFN +DTT, *Lane 4:* PEG_{10}-IFN -DTT, *Lane 5:* PEG_{10}-IFN +DTT. **(D)** *Lane 1:* Novex pre-stained markers, *Lane 2:* IFN-PEG_{20}-IFN -DTT, *Lane 3:* IFN-PEG_{20}-IFN +DTT, *Lane 4:* PEG_{20}-IFN -DTT, *Lane 5:* PEG_{20}-IFN +DTT. The disulfide re-bridged conjugates were found to be stable to de-conjugation in DTT for 10 min at 90ºC.

Figure 7

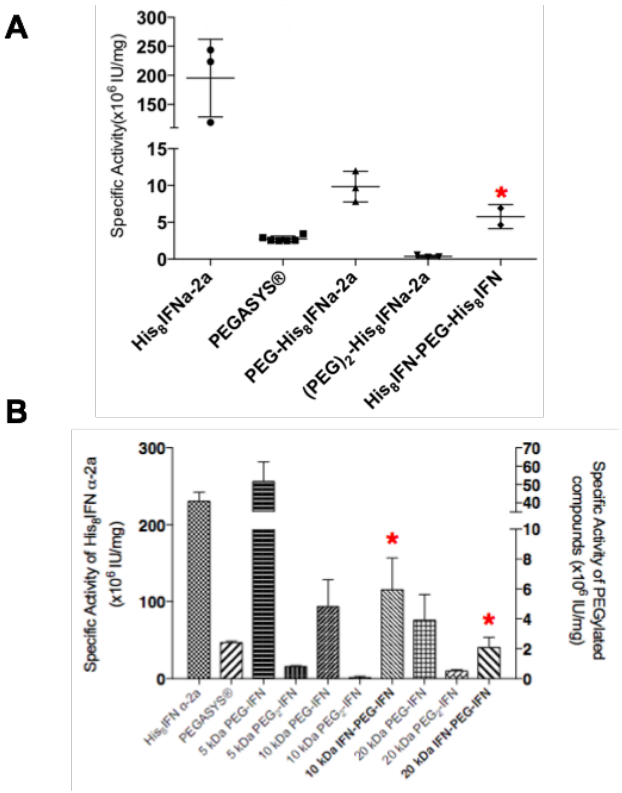

Figure 7. *In vitro* antiviral activity of conjugates with A549 cells incubated with EMCV virus. **(A)** His_8-tag modified conjugate and **(B)** disulfide re-bridged conjugates with the left hand axis to be used for the starting His_8IFN only. The right hand y-axis is used for all of the conjugates and the red stars indicate the IFN homo-dimers.

Table

Table 1

	Sample	Work-up	Yield (%)	Specific activity (MIU/mg) (n)
1	His_8IFN	-	-	231.31 ± 10.95 (3)
2	Pegasys®	-	-	2.43 ± 0.06 (3)
3	His_8IFN-PEG-$_{20}$-His_8IFN	IEX, SEC	1-2	5.78 ± 1.62 (2)
4	$(PEG_{20})_2$-His_8IFN	2× IEX	7	0.38 ± 0.16 (3)
5	PEG_{20}-His_8IFN	2× IEX	18	9.84 ± 2.08 (3)
6	IFN-PEG_{20}-IFN	IEX, SEC	1	2.12 ± 0.64 (4)
7	PEG_{20}-IFN	IEX	29	3.95 ± 1.69 (5)
8	$(PEG_{20})_2$-IFN	IEX	12	0.55 ± 0.04 (3)
9	IFN-PEG_{10}-IFN	IEX, SEC	1	5.99 ± 2.08 (4)
10	PEG_{10}-IFN	IEX	14	4.86 ± 1.77 (5)
11	$(PEG_{10})_2$-IFN	IEX	16	0.12 ± 0.04 (3)
12	PEG_5-IFN	IEX	29	51.89 ± 10.45 (2)
13	$(PEG_5)_2$-IFN	IEX	35	0.84 ± 0.03 (3)

Table 1. Summary of purification, yield and specific activities achieved for the IFN-PEG-IFN homo-dimers (in red) and the controls that were prepared. Grey shading used to indicate the His_8-tag conjugated species that were prepared. The remaining conjugates were prepared by disulfide re-bridging conjugation.

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