

Introduction

In recent years, poly(ethylene glycol) (PEG) conjugation (PEGylation) have been shown to confer superior clinical useful properties to proteins of therapeutic interest such as better physical and thermal stability, greater protection against proteolytic degradation, higher solubility, longer in vivo circulating half-lives, and lower clearance, thus enhancing efficacy. Additional qualities of pegylated proteins are reduced immunogenicity and antigenicity, as well as reduced toxicity.

To couple a monofunctional PEG (mPEG) moiety to a protein it is first necessary to activate the polymer by converting the hydroxyl terminus to some functional group capable of reacting with the functional groups found on the surface of proteins. The most common method has been to activate the PEG moiety with functional groups suitable for reaction with protein amino groups.

To evaluate the potential of different "activation" chemistries we have conducted comparative studies of synthesis of linear and branched mPEG derivatives with various MW's, developed novel chromatographic methods for their purification and tested some of these compounds for the PEGylation of **interferon alpha 2a (IFN2a)** and of **granulocyte colony stimulating factor (G-CSF)**

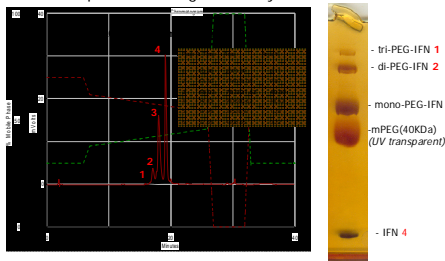
Polyethylene glycol chemistry

We developed novel chemical methods for the preparation of various mPEG molecules for conjugation:

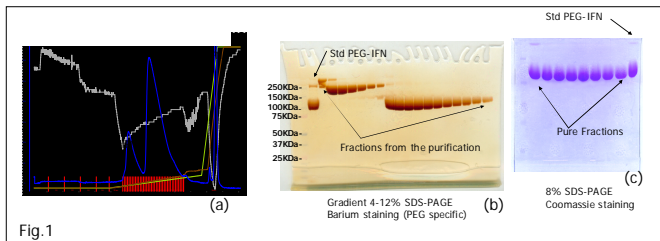
- mPEG active esters (4-nitrophenyl-, hydroxysuccinimidyl-, benzotriazolyl-) with MW from 3000 to 30000
- branched-PEG (based on diamino-carboxylic acids) with MW from 10000 to 40000Da
- Purification of these branched-PEG by various chromatographic techniques and their transformation in hydroxysuccinimidyl esters.
- protected mPEG-aldehydes (MW=3000-30000)
- mPEG-carboxylates (MW 3000 -30000): mPEG-OX (X= (CH₂)_nCOOH; n=0-3)

IFN Alpha 2a PEGylation using a branched 40 KDa mPEG-COOSu derivative

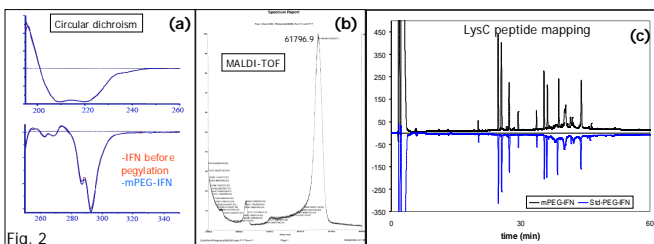
Reaction parameters: mPEG-COOSu stability/activity, mPEG excess, time, temperature, pH to minimize multimers and preserve biological activity of both mono-PEG-IFN and non-reacted-IFN.



Mono mPEG-IFN alpha2a purification and quality control

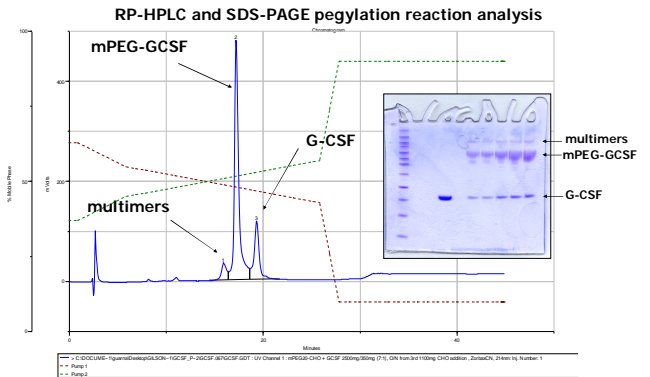


The mono-pegylated IFN alpha 2a is purified by LC (Fig. 1a) and the fractions checked by SDS-PAGE (Barium staining and Coomassie staining, Fig. 1b and 1c)

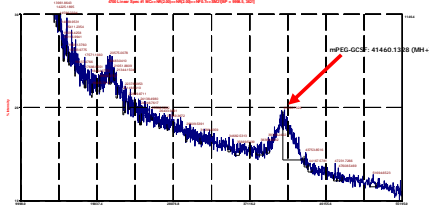


The quality controls confirm the purity and quality of the monopegylated IFN also in comparison to a standard of pegylated IFN: RP-HPLC (not shown), CD (Fig. 2a), MALDI-TOF (Fig.2b), peptide mapping (Fig. 2c), positional isomers composition (Fig. 2d). The *in vitro* bioassay returns bioactivity values perfectly in range with the values of standard pegylated IFN (~3% of non-pegylated IFN).

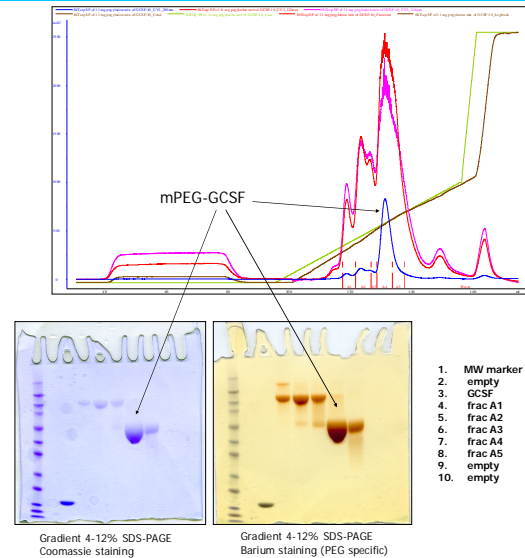
G-CSF PEGylation using a linear 20KDa mPEG-CHO derivative



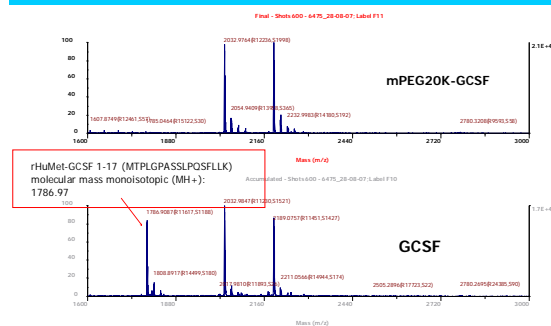
MALDI-TOF pos. linear mode of the main fraction from RP-HPLC



mPEG-GCSF purification and quality control



MALDI-TOF analysis of a trypsin digest of 20 ug aliquots of GCSF and mPEG20K-GCSF demonstrates that the site of mPEG attachment is the N-terminus of the molecule



Conclusions

We established scalable protocols for the synthesis of activated linear and branched mPEG derivatives with good purity and yields. The activated derivatives were efficient in tests of pegylation of IFN alpha 2a and G-CSF giving monopegylated molecules with characteristics comparable to commercial standards.