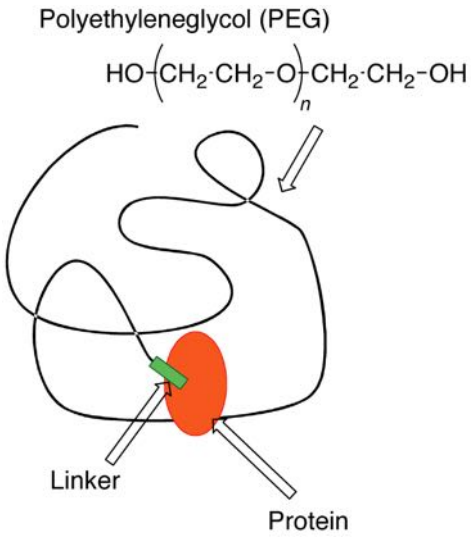
Protein Modification

March 17, 2015

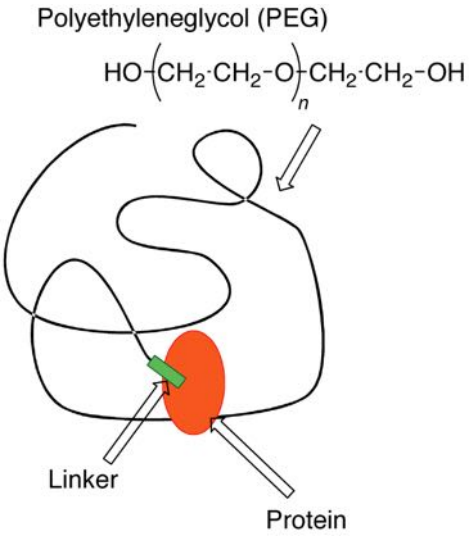
Protein Conjugation

- The unique specificity and potency of proteins and peptides indicate promising applications as therapeutics.
- Share common shortcomings:
  - ⇒ Short circulating half-life.
  - ⇒ Potential for immunogenicity.
  - ⇒ Susceptibility to proteolytic degradation.
  - ⇒ Low solubility.
- One solution is fusion or conjugation of the protein to natural or synthetic polymers.



Protein Conjugation

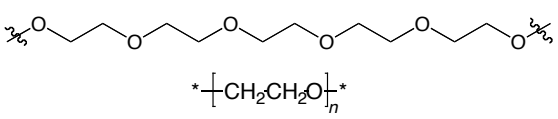
- Conjugation to natural or synthetic polymer:
  - ⇒ Increase apparent size of peptide or protein (reduces renal clearance).
  - ⇒ Shield antigenic epitopes.
  - ⇒ Reduce degradation by proteolytic enzymes.
  - ⇒ Improve solubility.
- Factors influencing these properties:
  - ⇒ Nature of the polymer (PEG/ Dextran).
  - ⇒ Degree of substitution.
  - ⇒ Molecular weight and structure of polymer.
  - ⇒ Site of substitution.
  - ⇒ Nature of linker segment.



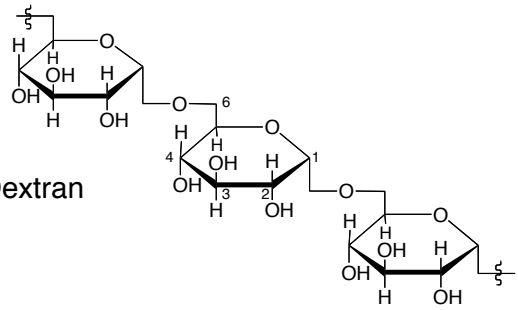
Polyethyleneglycol and Dextran

- The two polymers used most frequently in protein modification are:
  - Polyethyleneglycol: a linear or branched polyether with a -CH_2CH_2O- repeat unit and terminating in -OH group(s).
  - Dextran: a linear or branched polysaccharide assembled from glucose residues [α(1→6) linkage with 1→2, 1→3 and 1→4 branches].
- Contribute to aqueous solubility and hydrophilicity.
- Both contain -OH groups for linking to proteins.

Polyethyleneglycol

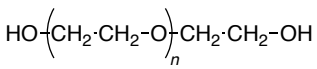


Dextran

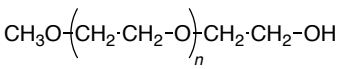


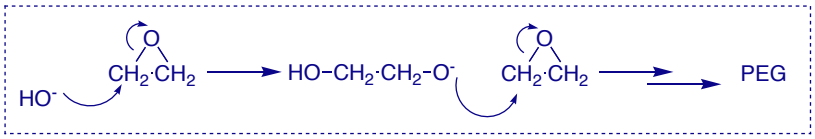
Polyethyleneglycol (PEG)

PEG (dihydroxy, diol)



mPEG (monomethoxy)

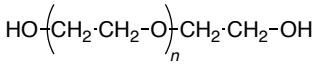




- Polyethyleneglycol refers to linear or branched polyethers.
- The most common forms terminate in hydroxyl groups.
- PEG is synthesized through anionic ring opening polymerization of ethylene oxide
- Generally initiated through nucleophilic attack by hydroxide or methoxide anions.
- Methoxide gives the monomethoxy variant (mPEG).
- Diol contamination in early syntheses of mPEG.

Polyethyleneglycol (PEG)

PEG (dihydroxy, diol)



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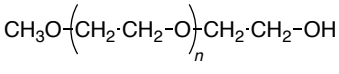
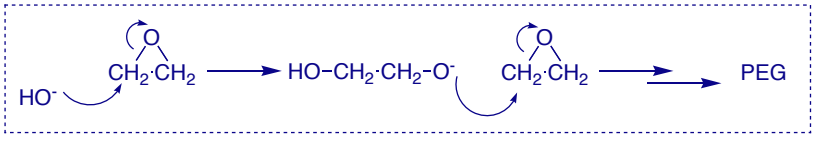




- PEG has a relatively narrow polydispersity (M_w/M_n), ~1.01 for low molecular weight PEGs (<50kD) to 1.1 for PEGS over 50kD.
- PEG is soluble in both organic and aqueous solvents allowing formation of conjugates under mild-physiological conditions.
- PEG is exceptionally well solvated by water (~2-3 water molecules per ethylene oxide unit)
- In solution PEG molecules demonstrate behavior consistent with proteins 5-10 times the actual size (mw) of the PEG molecule.
- It is believed that these properties contribute to the ability of PEG to reduce immunogenicity, prevent enzymatic degradation and reduce elimination of the peptide conjugate via the kidneys.

Chemistry of Pegylation

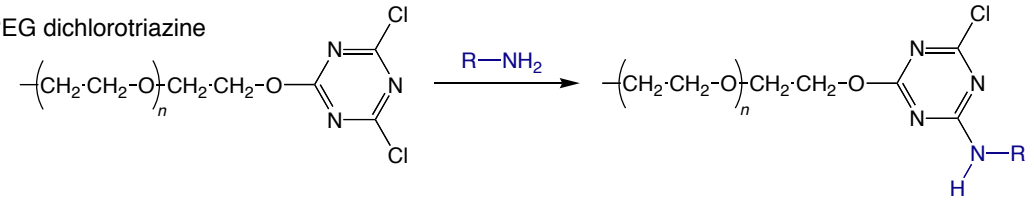
- Proteins present a range of functional groups that provide avenues to conjugation.
  - ⇒*N*-terminus (-NH_3^+), Lys (-$^{\varepsilon}NH_3^+$)
  - ⇒His (imidazole NH), Arg (guanidinium).
  - ⇒*C*-terminus (-CO_2^-), Asp (-$^{\beta}CO_2^-$), Glu (-$^{\gamma}CO_2^-$).
  - ⇒Cys (-SH)
  - ⇒Tyr (-OH), Ser (-OH) and Thr (-OH).

Chemistry of Pegylation

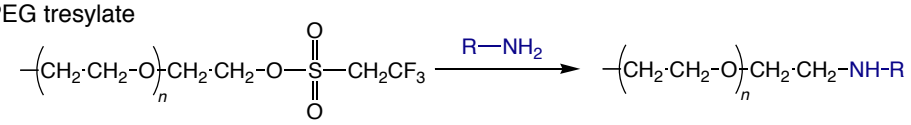
- Functional groups on PEG are activated.
- Primary focus for conjugation has been the N-terminal an Lys side chain amino groups.
- Potential for a large number of positional isomers.
- The benefits of PEG conjugation (PEGylation) are influenced by the size of the PEG groups, the degree of substitution, and the location of the linkage.
- Historically, PEGylation results in a heterogenous mixture of PEGylation products.

PEG: First Generation

PEG dichlorotriazine



PEG tresylate



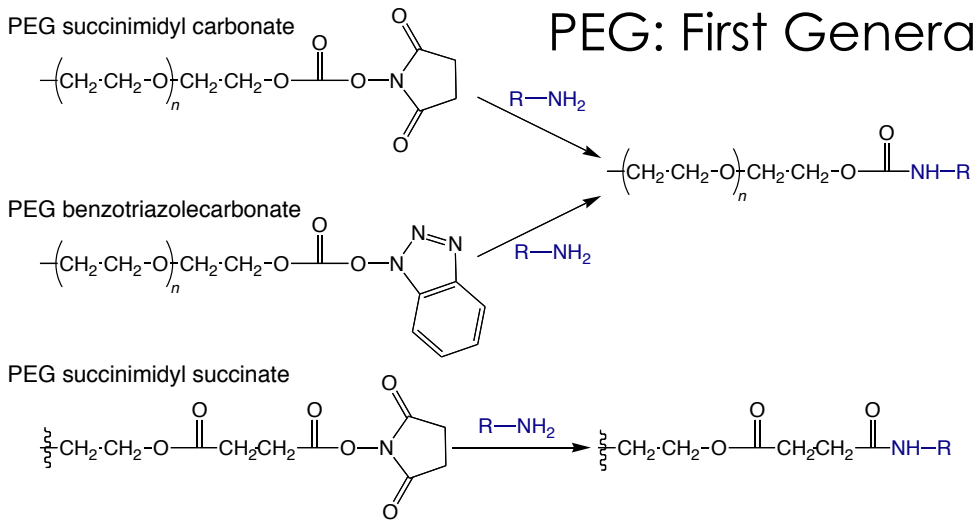
- Coupling of PEG to peptide/protein requires mild reaction conditions.
- Mainly focused on *N*-terminal and Lys side chain amino groups.
- PEG impurities limited process to low mw PEG.
- Low selectivity in sites and degree of modification presented problems

PEG: First Generation

PEG succinimidyl carbonate

PEG benzotriazolecarbonate

PEG succinimidyl succinate



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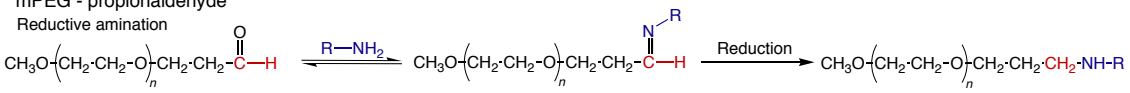
PEG: Second Generation

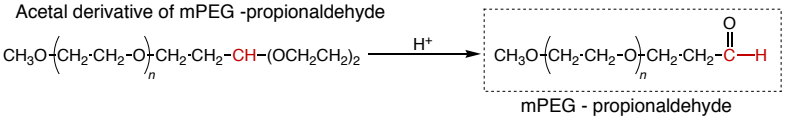
Amino Groups

mPEG - propionaldehyde

Reductive amination

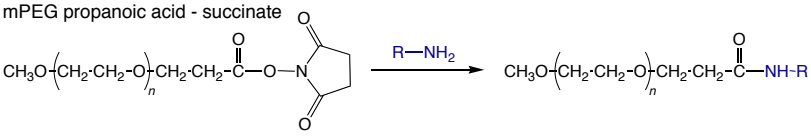


Acetal derivative of mPEG -propionaldehyde



mPEG - propionaldehyde

mPEG propanoic acid - succinate



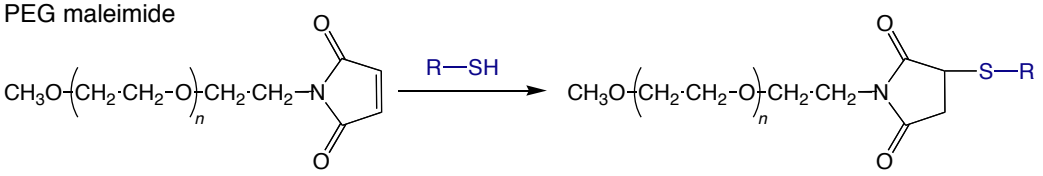
- PEG chemistry has evolved such that diol contamination and molecular weight restrictions have been minimized.
- The linkage chemistry has become more selective and the resulting linkages more durable.
- One early strategy capitalized on reductive amination.
- Active esters of PEG carboxylic acids are commonly used to form amide bond linkages .

PEG: Second Generation

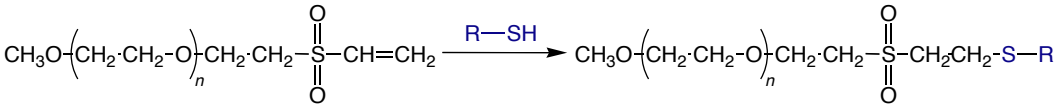
Thiol Groups

- Free Cys residues on the protein surface are the primary targets for site specific modification.
- There are fewer Cys residues present on the surface than there are Lys.
- **One strategy is to form stable thioether linkages**.
- Another is to form disulfide linkages.

PEG maleimide



PEG vinyl sulfone



PEG: Second Generation

Thiol Groups

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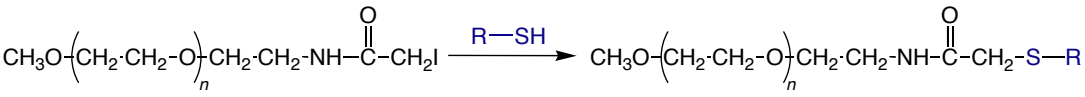
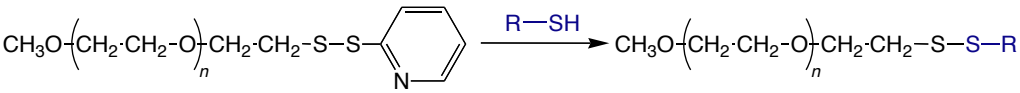
- **Another is to form disulfide linkages.**

PEG iodoacetamide



PEG orthopyridyl disulfide



PEG: Second Generation

Labile Linkages

- Most pegylation strategies utilize stable linkages.
- Stable linkages have been known to affect peptide/ protein activity.
- PEG molecular weight and structure can affect activity.
- One approach to recovering activity lost due to pegylation is to utilize linkage chemistries that release the protein over time.

  ⇒Enzymatic degradation.

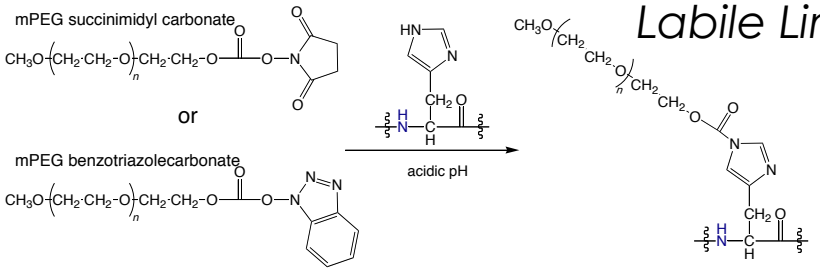
  ⇒Hydrolytic cleavage.

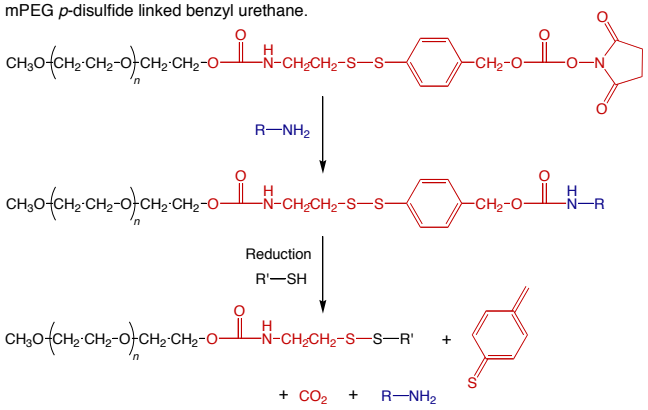
  ⇒Reductive cleavage.

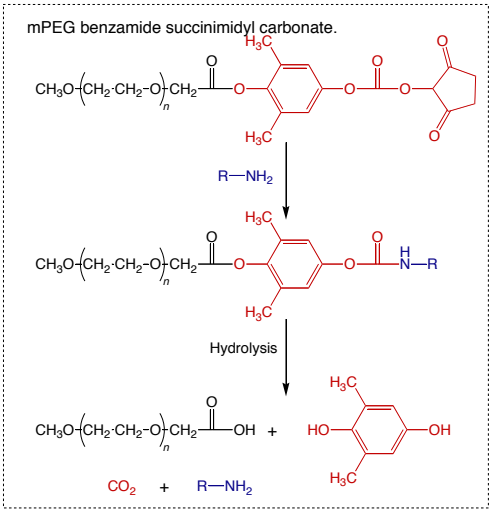
∗ Performance in *in vitro* experiments does not necessarily correlate with *in vivo* activity.

PEG: Second Generation

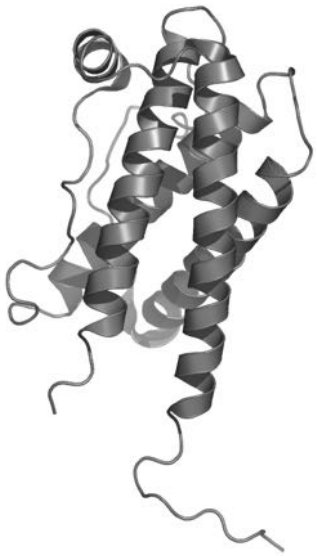
Labile Linkers

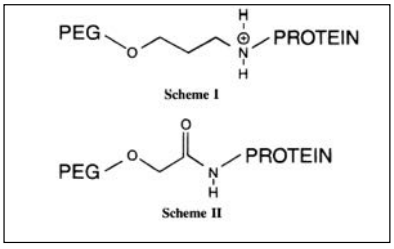






PEGylated G-CSF





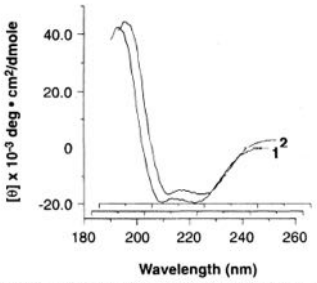


Fig. 5. Circular dichroism spectra of the PEGylated rhG-CSF conjugates. Far ultraviolet circular dichroism spectra of alkylated PEG-rhG-CSF conjugate (spectrum 1) at 0.9 mg/ml, and acylated PEG-rhG-CSF conjugate (spectrum 2) at 1.2 mg/ml. All samples were in 1 mM HCl at 23°C.

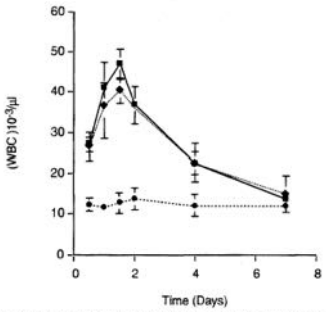
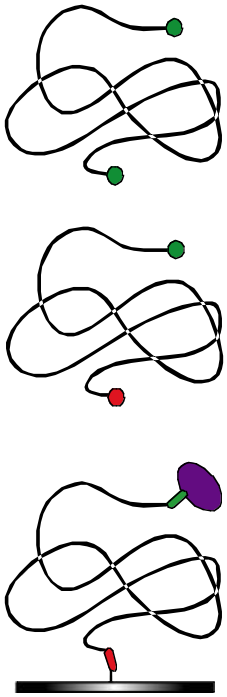


Fig. 4. The induction of peripheral WBC counts in hamsters from the subcutaneous injection of either vehicle (buffer) only consisting of 10mM sodium acetate, pH 4.0, containing 5% sorbitol (●), PEGylated rhG-CSF (◆) from the acylation conjugation method or PEGylated rhG-CSF (■) from the alkylation conjugation method and measured over time. The data represent the mean +/− S.D. for n = 4 hamsters/point.

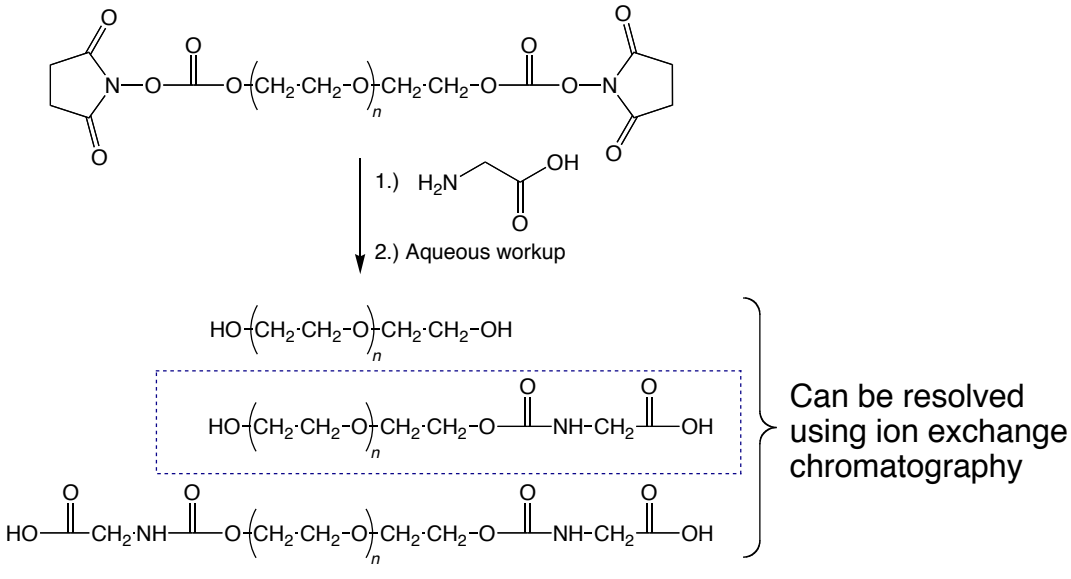
PEG: Asymmetric Substitution

- Heterobifunctional/ asymmetric PEG's are PEG's bearing dissimilar terminal groups.
- Such constructs could be used to link two different chemical entities. (i.e. immobilization of proteins on surfaces).
- Preferred end groups include: NHS-esters, maleimide, vinyl sulfone, pyridyl disulfide, and amino and carboxylic acid groups.



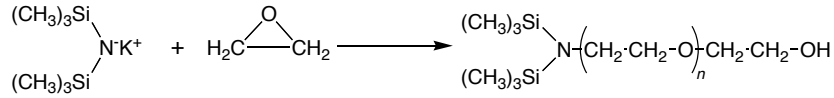
PEG: Asymmetric Substitution

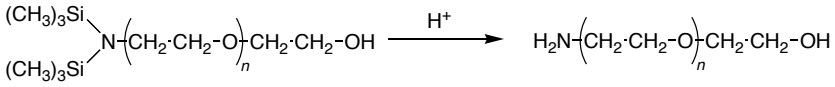
Derived from PEG succinimidyl carbonate



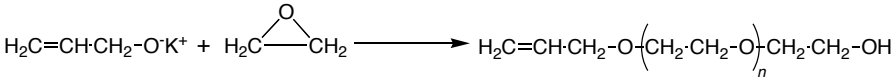
PEG: Asymmetric Substitution

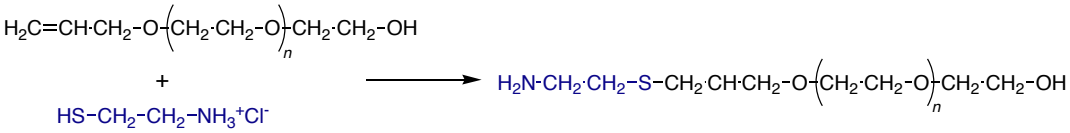
Using a bis(trimethylsilyl)amide initiator



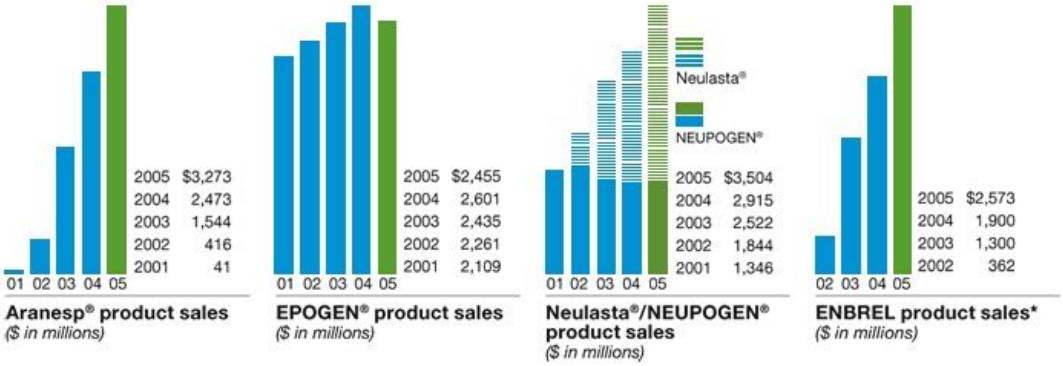


Using an allyl alcoholate initiator





Protein Therapeutics



Amgen 2005 Annual Report:
http://www.amgen.com/investors/AnnualReport2005/financials_review.html

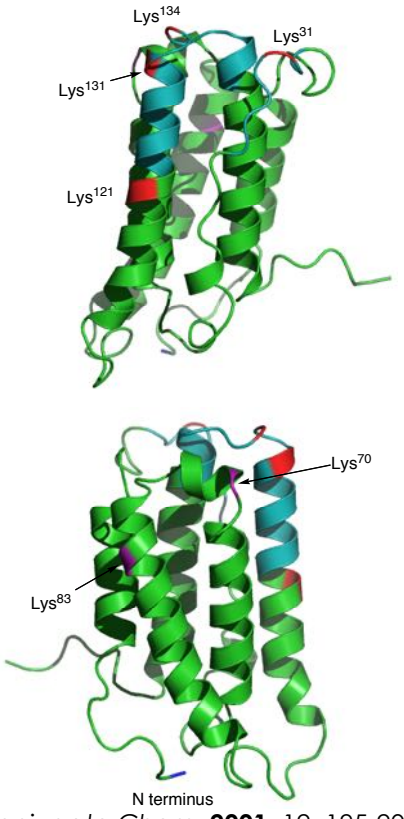
Hepatitis C Virus (HCV)

- Hepatitis is a general term used to refer to inflammation of the liver, and viral hepatitis refers to hepatitis that is caused by viral infection.
- Chronic viral hepatitis C (HCV) is a major health problem, and is the leading cause of cirrhosis of the liver.
- HCV infects liver cells and can cause severe inflammation of the liver.
- The disease is usually spread by sharing infected needles with a carrier, receiving infected blood products, or accidental exposure to infected blood.
  - ~60% of those infected become chronic carriers
  - ~20% of those with chronic infection develop cirrhosis.
  - Up to 20% of those with cirrhosis develop liver cancer.
- It is estimated that ~3% of the the world population have HCV.

http://www.who.int/csr/disease/hepatitis/whocdscsrlyo2003/en/index1.html

Interferon α

- Interferon a refers to a family of closely related proteins, which are:
  - Anti-viral
  - Anti-tumor
  - Immunomodulatory
- The human interferons INF α-2a and INF α-2b have been developed into drugs for the treatment of viral infections and cancer.
- These proteins are cleared rapidly by the body with terminal half-lives of 4-8h.
- Sustained therapeutic benefit requires frequent treatments.



Bioconjugate Chem **2001**, 12, 195-202.

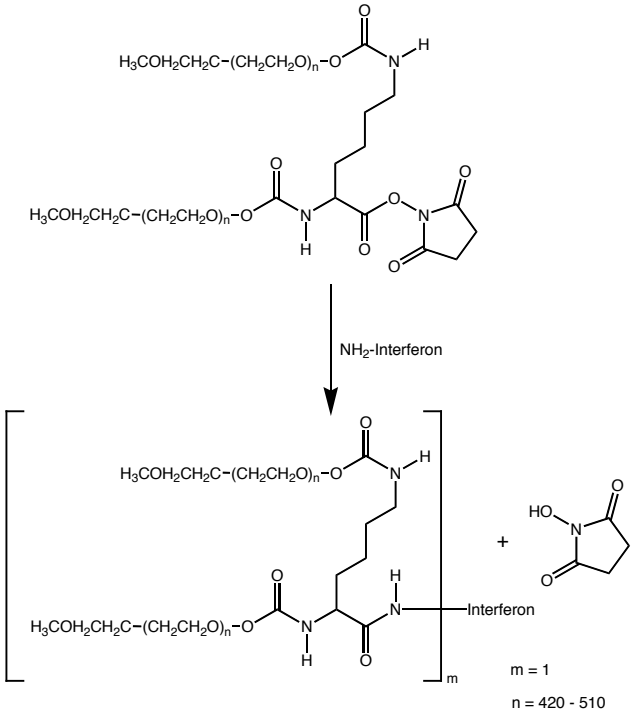
Pegylation: Insights

- In 1994, an effort to develop a PEG-INF α-2a was terminated due to poor performance in clinical trials.
- New insights into pegylation:
  1) *In vitro* activity in some cases is not necessarily indicative of *in vivo* performance.
  2) A single large PEG group is better than several smaller PEG groups.
  3) Linear PEG distributes throughout the body.
  4) Branched PEG is less widely distributed and early on is delivered to the liver and the spleen.
  5) Branched PEG derivatives show greater stability and robustness.
  6) Smaller linear PEG derivatives may deposit in kidney vacuoles.

Bioconjugate Chem **2001**, 12, 195-202.

Pegylation of INF α-2a

- New approach used a 40kd branched PEG (PEG_2).
  - Two 20kD mPEG chains.
  - Attached to ε- and α-amino groups of Lys via urethane linkages.
- Conjugated to free INF α-2a using the succinimido ester of the Lys carboxyl group.
  - 45-50% mono substituted.
  - 5-10% multiple PEG groups.
  - 40-50% unmodified



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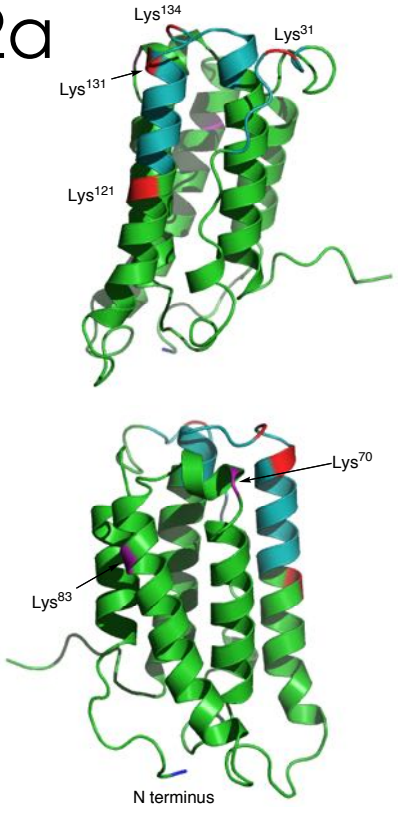
Pegylation of INF α-2a

- Estimate mw of the PEG_2-IFN α-2a derivative to be ~59.2KD (~40kD avg mw of PEG_2 and 19.2 kD mw of INF α-2a).
- PEG_2-IFN α-2a appears larger by PAGE (96kD).
- Analysis of PEG_2-IFN α-2a and peptide fragments. indicated that in each case one of four Lys residues were the predominant sites of attachment (Lys^{31}, Lys^{121}, Lys^{131} and Lys^{134}).
- Some attachment at Lys^{70} and Lys^{83} and none at N-terminus.



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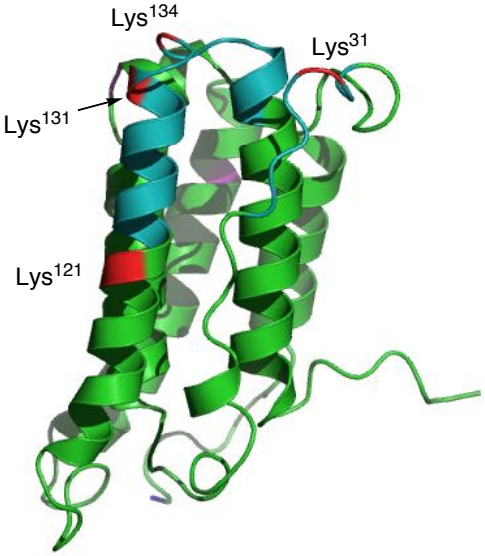
Degree of Modification

- In cell-culture bioassays, PEG_2-IFN α-2a demonstrates a potency that is 7% of that of unmodified IFN α-2a.
- Assay measures inhibitory effect on virus-induced cell lysis (bovine kidney cells challenged with vesicular somatitis virus.



Protein	anti-viral activity (IU/mg)	residual activity (%)
IFN α-2a	$2x10^8$	100
PEG_2-IFN α-2a	$1.4x10^7$	7

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In vitro vs. *in vivo* Performance

- In vivo anti-tumor activity.
- Human tumor cells implanted into mice.
- At all dosage levels, PEG_2-IFN α-2a showed significant reduction in tumor size.
- Underivatized IFN α-2a did not perform as well.
- 80 days after treatment, PEG_2-IFN α-2a treated mice had no tumors.

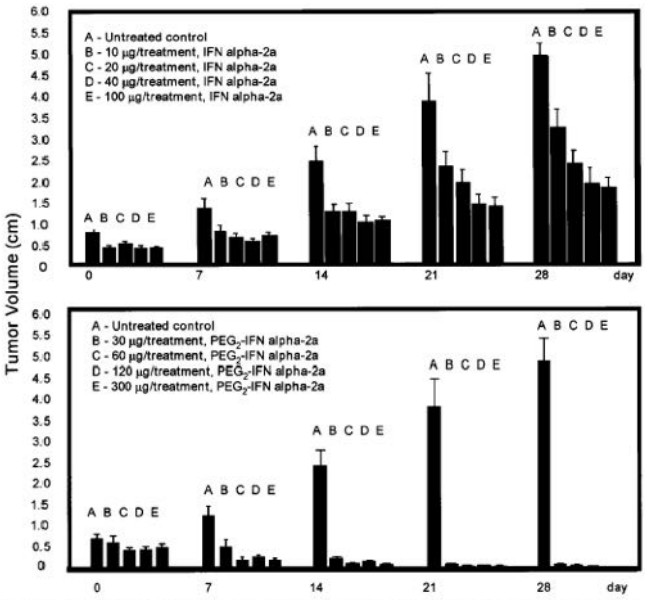

Figure 4. In vivo antitumor activity of interferon α-2a (top panel) and PEG_2-IFN (bottom panel) in athymic nude mice subcutaneously implanted with human renal A498 cells. Insert shows the amount of interferon α-2a and PEG_2-IFN used in the treatment of the mice implanted with the tumor; *x*- and *y*-axes indicate the days and the corresponding tumor volumes, respectively.

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In vitro vs. *in vivo* Performance

- Pharmacokinetics of PEG_2-IFN α-2a were determined (rats).
- Serum activity of IFN α-2a peaked after 1h after subcutaneous injection.
- Peak activity of PEG_2-IFN α-2a occurred 24h after injection.
- PEG_2-IFN α-2a demonstrated a 70 fold increase in serum half life.
- PEG_2-IFN α-2a has much greater plasma exposure.

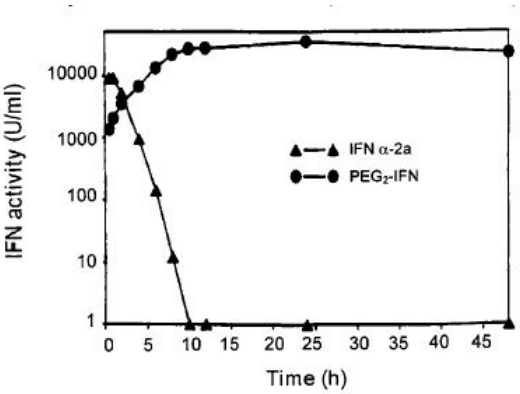

Figure 5. Mean serum activity versus time of interferon α-2a and PEG_2-IFN after subcutaneous injection in rats. PEG_2-IFN has a 51-h half-life compared to 0.7-h for interferon α-2a concomitant with mean plasma residence time of 80 and 1.6 h, respectively. Logarithmic regression analyses were used to calculate the pharmacokinetic parameters.

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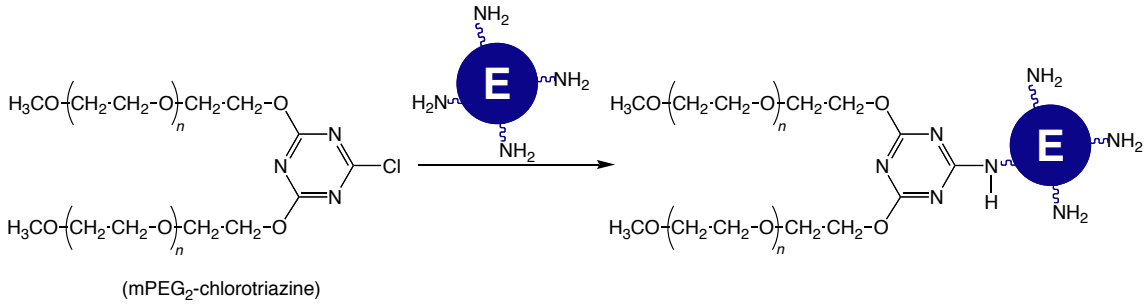
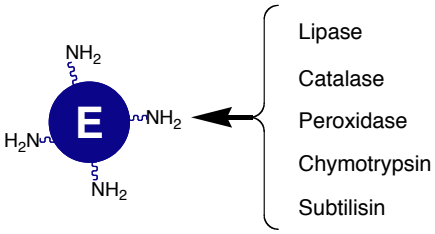
PEGylation of enzymes using a 2,4-bis(mPEG)-6-s-chlorotriazine

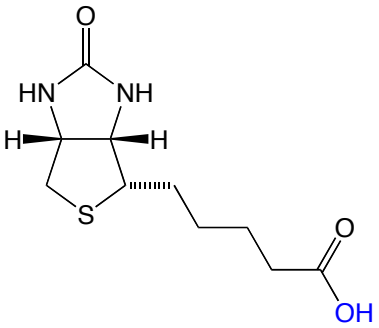


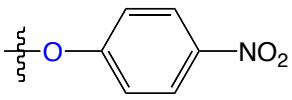


Biotinylation

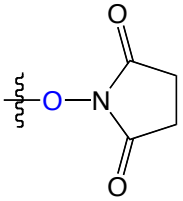
- Protein conjugation is not limited to polymers and macromolecules.
- Biotinyation is a common protein modification.
- Biotin is a coenzyme in fatty acid metabolism and is involved with other processes.

Biotin





p-nitropheynyl ester



succinimido ester

Streptavidin

- Streptavidin is a tetrameric protein (single subunit shown here).
- Binds biotin with high affinity (K_d of ~10^{-14} M) – one of the highest reported.