Controlled Release of Proteins through Releasable PEGylation

Hong Zhao*, Karen Yang, Cliff Longley, Shu-Min Liu, Yixian Zhang, Qing-Hong Dai, Jing Xia, Dechun Wu, Charles Conover

Enzon Pharmaceuticals, Inc., 20 Kingsbridge Road, Piscataway, NJ 08854

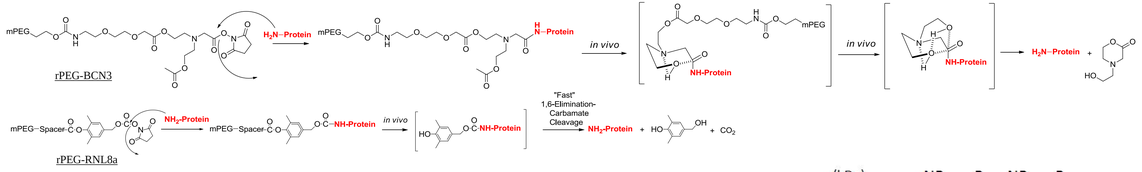
email: hong.zhao@enzon.com

Introduction

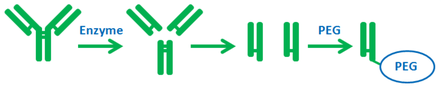
PEGylation is a proven drug delivery technology. Currently there are 11 PEGylated therapeutics on the market which are permanently PEGylated. Diminution of biological activity of both enzymes and protein is commonly encountered following permanent bioconjugation with PEG polymers. Therefore, recent research has examined the potential development of releasable PEGylation[1, 2], wherein covalent attachments of PEG polymer strands are shed via a controlled release mechanism derived from a degradable linkage at the attachment site on the protein surface. Releasable PEGylation may offer the greatest benefits to smaller-sized proteins such as antibody fragments (Fab, scFv, and Fv), cytokines, peptides, and small molecules.

Cytokine molecule IFN-β-1b and anti-TNF-α Fab were used as model compounds and we focused the current studies on BCN and RNL releasable PEG conjugates[3]. Our investigations of the comparative pharmacokinetics of native IFN-β-1b and PEGylated IFN-β-1b compounds demonstrate a new paradigm for interferon therapy through controlled release. At the same time, the ability to maintain binding affinity and biological activity of released Fab provides an alternative approach to prolong the half-life of antibody fragments without reengineering the protein.

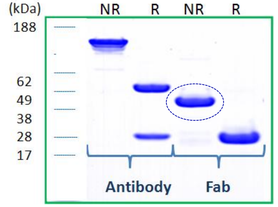
Releasable PEGylation of Antibody Fab



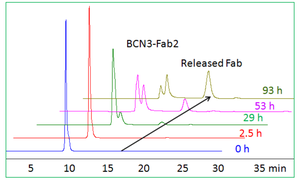
Compound	Linker	PEG mass (kDa)	PEG # (SEC-MALS)
BCN3-Fab-1	R	30	1-3
BCN3-Fab-2	R	30	7-8
RNL8a-Fab-1	R	30	4-5
RNL8a-Fab-2	R	30	10-11
SC-Fab-1	P	30	1-3
SC-Fab-2	P	30	9-10

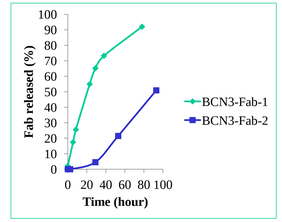


- Remicade Fab produced by enzyme digestion was PEGylated with BCN3 or RNL8a at different reaction molar ratios of PEG to Fab. (Permanent PEG SC was employed as a no-release control). PEG-Fab-1 was purified by cation exchange column and PEG-Fab-2 was isolated by SEC column.
- The number of PEG attached per Fab was analyzed with SEC-MALS.



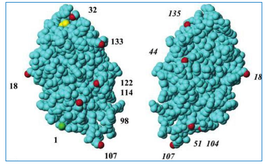
Analysis of Released Antibody Fab by SEC-HPLC





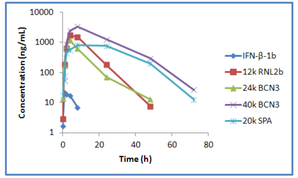
- Release of Fab from PEG-BCN-Fab conjugates was conducted in PBS, pH 7.4, 37°C At the times indicated, an aliquot was withdrawn and percent Fab released was analyzed on SEC-HPLC.
- Control with permanently PEGylated SC-Fab did not show any release under the same release conditions (data not shown).

Pharmacokinetics of PEG-IFN-β-1b



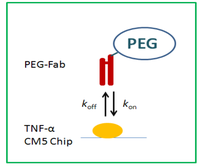
Two views of the human IFN-β model with exposed lysines (PDB file Name 1AU1). The space-filling model is shown from two different angles, rotated by 180°. The 11 lysines are numbered and highlighted with nitrogen in red. The N-terminal serine-1 amino group is green and the disulfide bond in yellow. Lysine-114 is not readily visible in this figure.

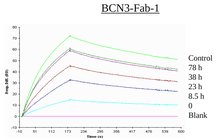
Compound	AUC (ng/mL•h)	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)
IFN-β-1b	226	1.29	1.38	21
RNL2b-12k	26235	4.47	6.46	1495
BCN3-24K	11248	1.87	4.31	961
BCN3-40k	74792	5.07	9.27	2967
SPA-20k	32610	8.93	12.90	930

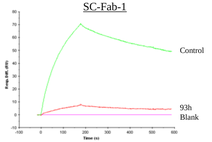


- The data showed that *sc* administration of compounds at 0.2 mg/kg in mice with both permanent and releasable PEGs resulted in a 50-fold to 330-fold increase in AUC compared with native interferon.
- Pharmacokinetics could be controlled by PEG#, PEG size, type of PEG linker. The longer circulating time could be achieved by employing slower releasable linker and by incorporating higher number of releasable PEG.

Biacore Binding Kinetics of BCN3-Fab-1 to TNF-α



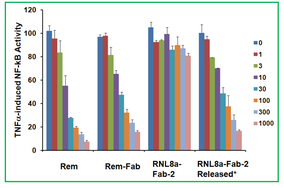


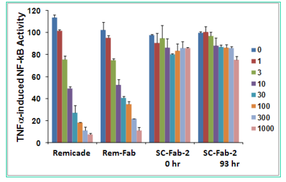


Sample	Time at 37°C (hour)	k_{on} (1/Ms)	k_{off} (1/s)	k_{on} (%)	k_{off} (%)	% Fab released
Fab	0	3.52E+05	7.39E-04	100	100	--
	78	3.57E+05	8.30E-04	101	112	--
BCN3-Fab-1	0	1.59E+05	9.14E-04	45	124	1
	8.5	1.07E+05	9.17E-04	30	124	26
	23	1.96E+05	9.07E-04	56	123	55
	38	2.76E+05	8.74E-04	78	118	73
	78	3.02E+05	8.79E-04	86	119	92

- BCN3-Fab-1 was incubated at 37°C in PBS, pH 7.4. At the times indicated, an aliquot was withdrawn and analyzed for TNF-α binding. Kinetic constants were obtained using a single concentration (25 nM) and 1:1 Langmuir fit. Native Fab was used as control.
- As Fab was released from BCN3-Fab-1, k_{on} increased and k_{off} did not change (k_{on} is concentration dependent and k_{off} is not). The amount of Fab released was analyzed by HPLC.
- Permanently PEGylated SC-Fab-1 bound weakly to TNF-α over the time of incubation.

Inhibition of TNF-α Cellular Activity by rPEG-Fab





Compound	Pre-incubation (T=0 hr) IC_{50} (ng/mL)	Post-incubation (T=93 hr) IC_{50} (ng/mL)	Post-incubation (T=93 hr) % Fab released (SEC-HPLC)
Remicade-Fab	31 (±2)	31 (±2)	——
RNL8a-Fab-2	>1000	38	42
BCN3-Fab-2	847	129	74
SC-Fab-2	>1000	>1000	0

Method:

PEG-Fab conjugates were incubated in Tris buffer (pH 8.5) at 37°C for 93 hours. HEK293-NF-kB-luc cells were plated for 16 hours and then treated with indicated compounds (ng/mL) for 10 min followed with 1 ng/mL of TNF-α. Luciferase activity was determined after 6 hours.

Results:

- Released Fab shows comparable activity to native Fab in the inhibition of TNF-α-induced NF-kB activity.
- Permanently PEGylated SC-Fab-2 has no anti TNF-α activity.

Conclusions

- **A series of releasable PEG-IFN-β-1b and PEG-TNF-α Fab conjugates with different releasable PEG linkers have been prepared.**
- **The releasable PEG linkers display suitable characteristics for applications in formulation of IFN-β-1b. The increase of protein exposure in animals is comparable to that of permanent PEGylation.**
- **Releasable PEG-Fab conjugates can release native Fab in buffers at different rates. The types of PEG linker and number of PEG strands can alter the rate of releasing.**
- **The released Fab had comparable binding affinity to TNF-α as analyzed by Biacore. Even at lower concentrations, the released Fab showed comparable activity to native Fab to inhibit TNF-α induced NF-kB cellular activity whereas the permanent PEG-Fab had no anti TNF-α activity.**
- **Releasable PEG linkers provide a practical option to improve protein pharmaceutical properties when reengineering of the protein molecules is not desired or feasible.**

References

1. **Filpula, D.; Zhao, H. *Advanced Drug Delivery Reviews*, 2008, 60, 29-49.**
2. **Greenwald, R. B., Zhao, H. *Prodrugs: Challenges and rewards. Springer*, 2007, Chapter 2.3.1, pp 283-338.**
3. **Zhao, H.; Yang, K.; Martinez, A.; Basu, A.; Chintala, R.; Liu, H. C.; Janjua, A.; Wang, M.; Filpula, D. *Bioconjugate Chem*. 2006, 17, 2, 341-351.**