

1. Despite many studies on the use of EGF in clinical wound healing and significant commercial investment in R&D for use of EGF, there are yet no viable clinical products using EGF for wound healing.

It has been proposed that it might be more efficacious to deliver EGF in a form where it cannot be internalized but can still be bound by EGFR; i.e., by tethering EGF to a solid (non-internalizable) substrate via a long, water soluble polymer chain which allows EGF to diffuse freely over some volume prescribed by the length of the tether (~70 nm) but which keeps it localized [see handout paper by Kuhl et al, Nat. Med.]. The use of scaffolds to achieve guided tissue regeneration makes this approach appear feasible from a development standpoint. Further, the unique chemical properties of EGF -- it has just one primary amine -- allow it to be covalently linked to a tether in a single conformation that should presumably be competent to bind the receptor.

(a) The molecule of interest for tethering EGF, polyethylene oxide (PEO), is widely used in biomaterials to inhibit protein and cell adhesion to surfaces. Because PEO is a highly swollen random coil in solution, there is concern that EGFR may not be able to bind EGF when it is tethered to PEO because PEO will sterically prevent access to the EGF molecule.

To test whether binding of EGF would be affected by the PEO tether, soluble conjugates comprising one EGF molecule linked to a tether were prepared and the receptor-mediated internalization properties were examined in the following experiment:

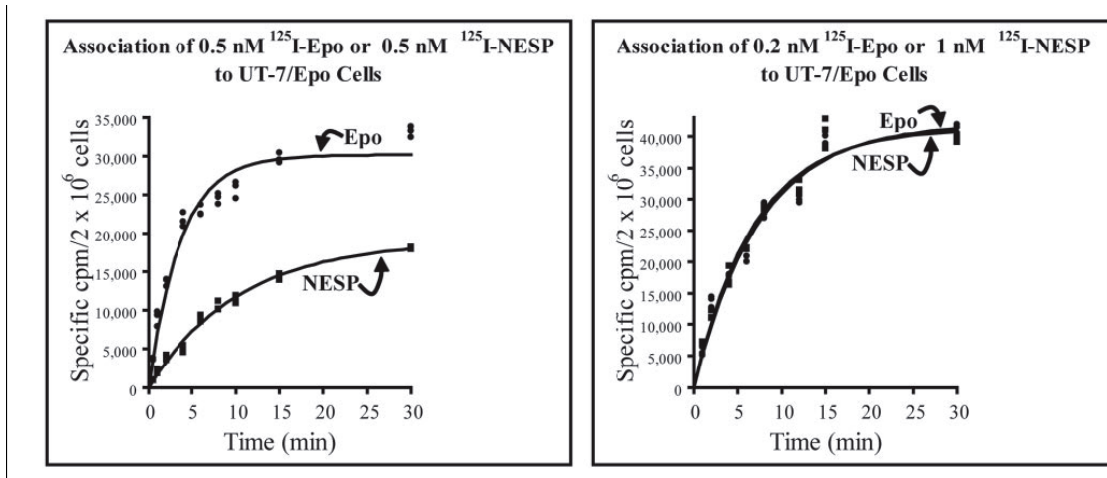
B82 cells were serum starved at 37°C for three hours before addition of ¹²⁵I-labeled EGF-PEO conjugate (16.4 pM). Parallel cultures were incubated for 1-20 minutes at 37°C and then transferred to an ice bath where the medium was removed and the cells washed twice in WHIPS buffer. *Surface radioactivity* was removed by washing the cells twice in acid strip solution, allowing the cells to incubate for 8 minutes in the acid strip during the first wash and 4 minutes during the second wash. *Internalized ligand* was then removed by solubilizing the cells in 1N NaOH.

The following data were obtained for the conjugate (error is $\pm 10\%$ on each measurement for 3 measurements):

TIME (MIN)	# SURFACE COMPLEXES	#INTERNAL COMPLEXES
2	88	82
4	100	78
6	117	89
8	111	156
10	116	158
12	119	217
14	170	348
16	186	447
18	207	568
20	264	782

The endocytic rate constant for soluble EGF on B82 cells has been reported as $k_{ec} = 0.15 \text{ min}^{-1}$. Based on these data, does it appear that the PEO tether significantly influences the binding of EGF to EGFR?

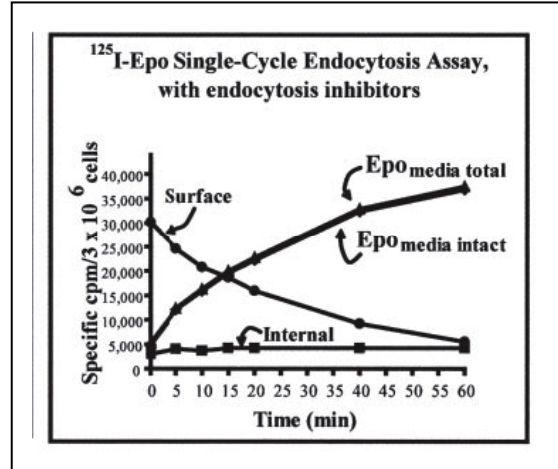
2. A hyperglycosylated version of EPO, “NESP” (Novel Erythropoiesis-stimulating protein), has a longer serum half-life than EPO but has a lower affinity for the EPO receptor. The mechanisms for this prolonged half-life have recently been illuminated.¹ The following data were obtained for binding of ¹²⁵I-labelled Epo or NESP to the surface of cells expressing Epo receptor under conditions where endocytosis was inhibited and where the concentration of ligand may be presumed constant at L_0 . The curve on the left compares binding of Epo and NESP when $L_{0, \text{Epo}} = L_{0, \text{NESP}} = 0.5 \text{ nM}$, and the curve on the right shows that equivalent association profiles are achieved when $L_{0, \text{Epo}} = 0.2 \text{ nM}$ and $L_{0, \text{NESP}} = 1 \text{ nM}$.



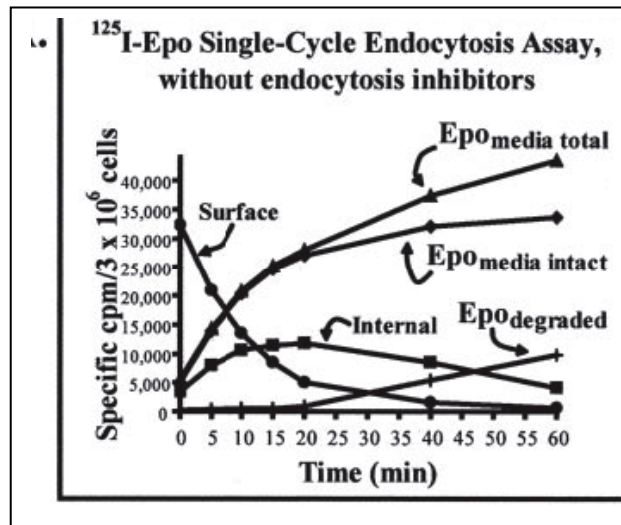
- (a) Compare the two curves for Epo, and explain in quantitative terms (i.e., using an equation) why the time required to reach equilibrium binding is longer for the data Epo on the right graph.
- (b) Estimate the relative values of K_D for Epo and NESP, and show the quantitative basis for your estimate.

¹ A.W. Gross & H.F. Lodish, J Biol. Chem., 281, 2024-2032 (2006).

- (c) After equilibrium binding of Epo is achieved (i.e. at 15 min of incubation in 0.5 nM Epo), the medium is removed from the cells and replaced with medium containing no Epo (but still containing endocytosis inhibitors). The amounts of Epo on the cell surface, cell interior, and culture medium are measured as a function of time and the data shown in the figure to the right were obtained. Explain the shape of the curve “surface”, and give an equation that describes it.



- (d) The experiment described in part c is now conducted in the absence of endocytosis inhibitors. Explain qualitatively the shape of the curve obtained for the amount of Epo inside the cell (“internal”) as shown in the data.



- (e) Analysis of NESP under the same protocols as described above shows that its internalization, degradation and recycling behavior appear similar to that obtained for Epo. In fact, quantitative analysis of the data using a full set of coupled linear differential

equations reveals that the rate constants for internalization, degradation, and recycling of NESP and Epo are identical in the cell types studied. The only observed difference between the two molecules is the cell surface K_D ; Epo binds faster than NESP ($k_{f, \text{Epo}} = 5 \times 10^8 \text{ M}^{-1}\text{min}^{-1}$ and $k_{f, \text{NESP}} = 1.1 \times 10^8 \text{ M}^{-1}\text{min}^{-1}$) and dissociates more slowly ($k_{r, \text{Epo}} = 0.029 \text{ min}^{-1}$ and $k_{r, \text{NESP}} = 0.042 \text{ min}^{-1}$).

Epo and NESP are used clinically at the same dose (same initial concentration in the blood). Propose the mechanism for the observed longer period needed between doses, and suggest the implications of this for expense of treatment.

3. Please download the files KinaseCascade.m, pset6_matlab.m, and ferrell.pdf. KinaseCascade.m is a model for part of the MAP-Kinase cascade, for the schematic drawn below. (Note this is an approximate model as the rate constants are all 1, Michaelis-Menten assumption is used everywhere and the K_m and V_{max} values are always the same! The initial conditions are based on real experimental values though.) The code pset6_matlab.m runs this model with different inputs, and you can modify this code for this problem.

The output in figure 1 from pset6_matlab.m depicts the output of activated RAF, MEK, and ERK at different input stimuli, and you can see that the further down in the cascade you look, the output has a greater and greater “switch-like response”. (Note this code will take a couple minutes to run!) The attached paper by Ferrell describes theoretically how an input stimulus in such a cascade can produce a switch-like output. The output in figure 2 from pset6_matlab.m depicts the output of activated ERK over time, for a large input stimulus.

- Would this system exhibit a switch-like response if there were no phosphatase enzymes in the cell for MEK and ERK?
- For the same large input stimulus used in figure 2 of the code, please consider the consequences of adding an inhibitor to this system. The inhibitor PD98059 binds to the inactive (completely unphosphorylated) form of MEK, preventing its phosphorylation by RAF, and has an IC_{50} of $2\mu M$. Please make a new model, like KinaseCascade.m, but with this inhibitor added. Please plot the steady-state (maximum) output of activated ERK for a range of inhibitor concentrations. How much inhibitor must you add for the level of activated ERK to be reduced by 90%? (Note, it's fine for you to approximate the rates for the inhibitor the same way all the other rate constants have been approximated in this model.)

