

Site-specific PEGylation of L-asparaginase II: effects on activity, stability and structure

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Asparaginases are therapeutic enzymes currently used for treatment of acute lymphoblastic leukemia and non-Hodgkin's lymphoma.^{1,2} Rapid serum clearance and immune inactivation are the main drawbacks that limit their application. This has been addressed by decorating the enzyme with biocompatible polymers. Specifically, a commercial formulation of *E. coli* L-asparaginase II randomly conjugated with mono-methoxy polyethylene glycol (mPEG) reduced the incidence of neutralizing antibodies and exhibited longer blood half-life activity than the non-conjugated enzyme. Unfortunately, random modification also impacts negatively on the drug's pharmacodynamics leading to drastically reduced biological activity (85 to 0%) and substrate affinity (K_M).¹⁻⁶ To improve this, herein we site-selectively modified asparaginase with bi-maleimide-PEGs by linking the polymers to cysteines which were introduced by mutagenesis. In addition, to reduce the number of polymers around the enzyme, we successfully cross-linked the asparaginase subunits to generate enzymatically active conjugates, using 1000, 2000 and 5000 g/mol PEGs.

We obtained different physical states (gel to soluble) and sizes (~140 to 400 kDa) for the conjugates, depending on the length of PEG used. The 1kDa-PEG-conjugate (gel) had a similar temperature enzymatic profile as a commercial randomly-PEGylated *E. coli* L-asparaginase II formulation (Sigma-Aldrich) and proved to be highly reusable. The 5kDa-PEG-conjugate (soluble) exhibited slightly higher enzymatic activity than the native non-conjugated enzyme when tested at 37°C, and 4.2 times more at 25°C. In addition, we obtained interesting data regarding expression of recombinant asparaginase cysteine-mutants, necessary for conjugation through thiol-maleimide chemistry. We found that our double-mutation (A38C-T263C) expressed at 33% compared to the native recombinant asparaginase, tested in *E. coli* BL21(DE3), and it was more complicated to purify using anion exchange chromatography. A mutant without the natural cysteine bond (C77-105S), still retained full enzymatic activity, but exhibited almost no extracellular secretory expression. Aiming to overcome this problem, we recently designed an expression system containing a His-tag along with deletion of the two-natural cysteines.^{7,8} This construct was relatively easy to purify, but again the secretory expression was very low. This let us to believe that the natural cysteine(s) in *E. coli* L-asparaginase II play a key role in the extracellular secretion mechanism of this enzyme.⁹

References:

[1] H Van den Berg. *Leukemia & Lymphoma* **2011** 52(2), 168-178. [2] VI Avramis, PN Tiwari. *International Journal of Nanomedicine* **2006** 1(3), 241-254. [3] Y Kodera, T Sekine, T Yasukohchi, Y Kiriu, M Hiroto, A Matsushima, Y Inada. *Bioconjugate Chemistry* **1993** 5(4), 283-286. [4] VI Avramis, S Sencer, AP Periclou, H Sather, BC Bostrom, LJ Cohen, AG Ettinger, LJ Ettinger, J Franklin, PS Gaynon, JM Hilden, B Lange, F Majlessipour, P Mathew, M Needle, J Neglia, G Reaman, JS Holcenberg. *Blood* **2002** 99, 1986-1994. [5] AL Soares, GM Guimarães, B Polakiewicz, R Nogueira de Moraes-Pitombo, J Abrahão-Neto. *International Journal of Pharmaceutics* **2002** 237, 163-170. [6] RJ Solá, K Griebenow. *BioDrugs* **2010** 24(1), 9-21. [7] MP Jennings, IR Beacham. *Journal of Bacteriology* **1990** 172(3), 1491-1498. [8] A Khushoo, Y Pal, BN Singh, KJ Mukherjee. *Protein Expression & Purification* **2004** 38(1), 29-36. [9] FJM Mergulhao, DK Summers, GA Monteiro. *Biotechnology Advances* **2005** 23(3), 177-202.

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