

# ExSAR<sup>TM</sup>

## ***EVALUATIONS OF STRUCTURAL COMPARABILITY BY HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY***

To explore research opportunities and research services,

Please contact:

Dawne Miller

[dmiller@exsar.com](mailto:dmiller@exsar.com)

ExSAR Corporation

11 Deer Park Drive, Suite 103,

Monmouth Junction, NJ 08852

Tel: 732-438-6500 Fax: 732-438-1919

# OVERVIEW

ExSAR's proprietary platform facilitates evaluations of structural comparability. The technology consists of an automated hydrogen/deuterium exchange platform (H/D-Ex) coupled to proprietary software for the rapid and reproducible measure of amide hydrogen exchange rates.

Due to its reproducibility, robustness and unique ability to detect subtle changes in structure, H/D-Ex has proven highly useful to those involved in process development. Whenever a change in formulation, synthesis, purification or handling is involved, and comparisons of protein structure need to be performed, H/D-Ex can be called upon to provide a reliable and moderately quick answer (2-3 day turn-around time).

In Section I are described technical aspects of the approach as well as a discussion on how these results are presented and what they mean. In Section II are described three case studies consisting of (i) a "Lot to Lot" comparison, (ii) a "Follow-on" product application and (iii) a structural stability sensing exercise. We believe the following information will be of much interest to an audience involved in process manufacturing and development. H/D-Ex should also be of interest to those generally involved in the development of follow-on biologics, as demonstrations of comparability will be necessary to secure abbreviated market approval pathways for their product.

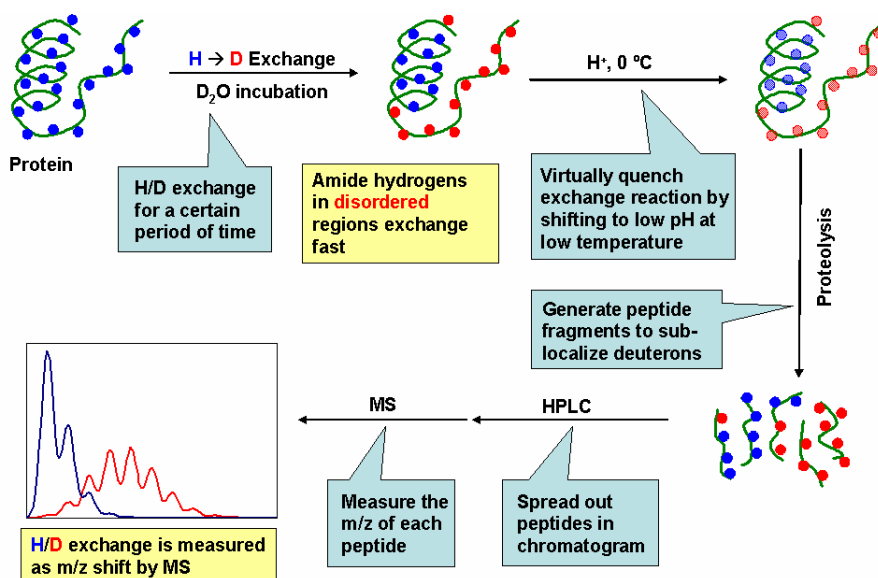
## A word on biologics and bioequivalence

Biologics, in contrast to conventional drugs, are large complex compounds often derived from living sources. Consequently, the manner of production possesses a certain degree of intrinsic uncertainty. For example the success of obtaining a properly folded recombinant protein can hinge upon the selection of an appropriate expression system as well as culture conditions. These choices can impact the existence and patterns of glycosylation and other post-translational modifications. A popular opinion held by policy makers at the FDA and by makers of innovative biologics, is that manufacturing and formulation processes must be faithfully reproduced or else the biologic cannot be deemed equally safe or efficacious<sup>1,2</sup>. A biogeneric and a marketed biologic which has undergone a change in formulation or manufacturing, are equally suspect of being non-bioequivalent to the original product. Bioequivalence is understood to mean as having the same safety and efficacy profile.

A bioequivalent product is likely to be structurally identical to its predecessor and be subject to similar trace aggregate and impurity levels. The take home message from the *Follow-on Biologics Workshop: Scientific Issues in Assessing the Similarity of Follow-on Protein Products*, NYAS, December 2005, is that no single analytical method will be sufficient to establish structural and compositional equivalence. For instance, H/D-Ex will not detect impurities and trace aggregates. On the other hand analytical techniques capable of detecting and quantifying components of a mixture, such as size exclusion chromatography with "in-line" mixed angle light scattering, do not provide the structural level of detail provided by H/D-Ex. Therefore demonstrations of equivalence will need to employ multiple analytical techniques, one of which should be H/D-Ex.

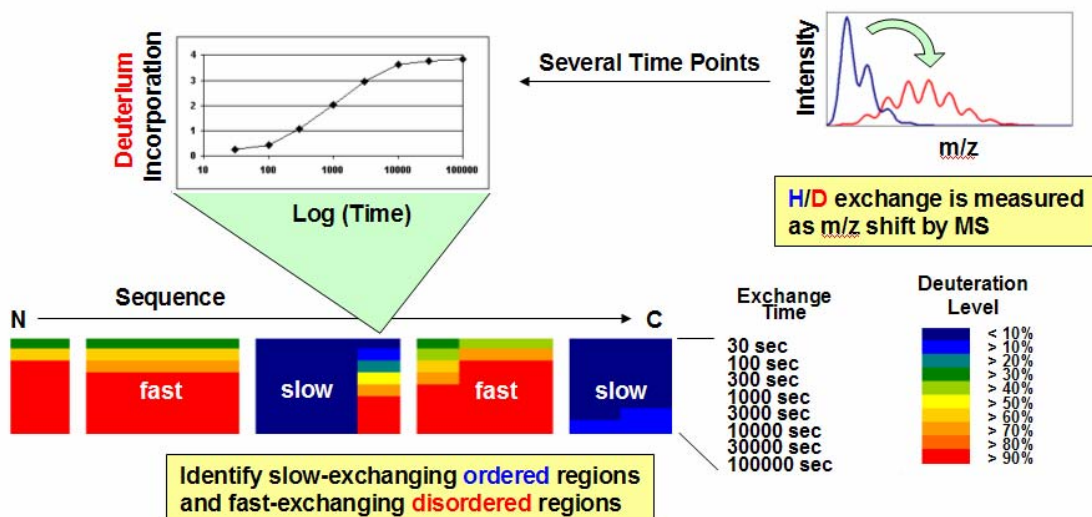
## I. Hydrogen/Deuterium Exchange Mass Spectrometry (H/D-Ex)

ExSAR's platform technology, based on hydrogen/deuterium exchange mass spectrometry (H/D-Ex), can be used to probe the conformational dynamics of a protein's 3D structure in solution. First, protein is mixed with deuterated buffer and incubated for a predetermined duration during which backbone amide hydrogens gradually exchange with bulk solvent deuterons. The exchange rate of each backbone amide hydrogen is unique to its environment; disordered regions and/or surface exposed regions exchange fast, ordered and/or buried regions exchange slow. Following the incubation period, the exchange reaction is essentially quenched by shifting the pH to around 2 while lowering the temperature to near 0°C. The exchanged protein is then proteolyzed with acid stable proteases. The peptic fragments are then chromatographically separated and their masses determined by mass spectrometry. The experiment is repeated in the absence of deuterium and the molecular weight difference of identical fragments attributed to deuteration. A schematic diagram representing ExSAR's H/D-Ex platform is illustrated in Figure 1.



**Figure 1.** H/D-Ex method overview

A deuteration curve will allow the rate of exchange to be calculated. This is accomplished by exposing the protein to deuterated solvent over increasing time periods. The experiment described in the preceding paragraph and illustrated above in Figure 1, is repeated for as many time points as necessary. Curves like the one illustrated in Figure 2 are generated by plotting deuteration levels as a function of on-exchange time. Deuteration trends can be difficult to recognize when looking at 30 or more curves. To tackle this problem, we've adopted the convention of representing these deuteration profiles in color. Deuteration levels are assigned a color spanning from red, highly deuterated, to blue, not highly deuterated. Then deuteration levels of various sequence segments are represented in the appropriate color and plotted beneath the corresponding sequence (see Figure 2). **These deuteration profiles are in essence a finger print of structure and dynamics.**



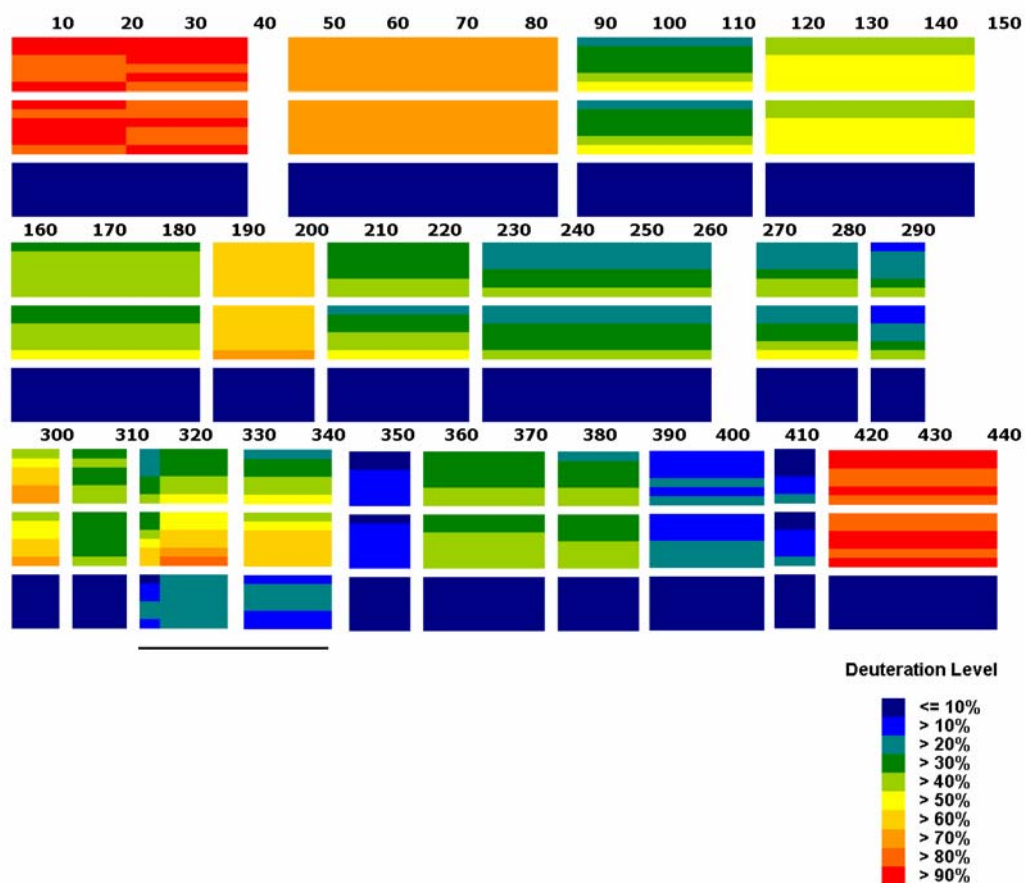
**Figure 2.** H/D-Ex is carried out over increasing exchange time periods. Results are represented as deuteration curves and global deuteration profiles.

## II. Biocomparability Case Studies

### Case study #1 – Lot to Lot Comparisons

The rate at which an amide hydrogen undergoes exchange is largely determined by the structural context in which that amide hydrogen resides, whether or not an amide hydrogen is involved in a hydrogen bond and the degree to which an amide hydrogen becomes solvent exposed. For example, an increase in exchange is understood as a region which has become more dynamic, more solvent exposed and less hydrogen bonded, typically due to the loss of regular secondary structure.

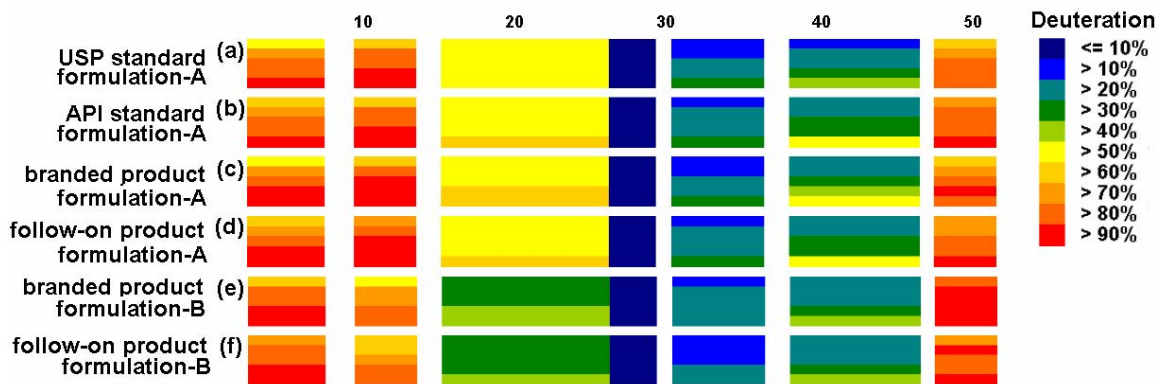
ExSAR recently identified a lot to lot difference in the protein dynamics of an antigen. The antigen was used by our client to generate therapeutic neutralizing antibodies. ExSAR identified a 20 residue region (underlined in Figure 3.) that exhibited a differential rate of amide hydrogen exchange in one of the analyzed lots. All lots were understood to contain exactly the same protein, and to have been equivalently manufactured. Our client was concerned about an unspecified modification and sought to gain additional data on equivalence. Indeed, a difference was detected in hydrogen exchange rates. This difference is indicative of a structure which has been compromised or simply changed. The proteins constituting these two lots have accordingly been deemed non-identical.



**Figure 3.** H/D-Ex patterns of two lots. Horizontal colored blocks represent analyzed peptic peptides. Numbering corresponds to the relative protein sequence position. The top two rows of colored blocks correspond to the deuteration levels of antigen-1 and -2 respectively. The difference in deuteration is shown in the bottom row. The deuteration level of each peptide is color-coded as shown for each of the six time points (15, 50, 150, 500, 1,500, and 5,000 s). The region showing non equivalence has been underlined.

## Case study #2 – Follow-on Product Applications

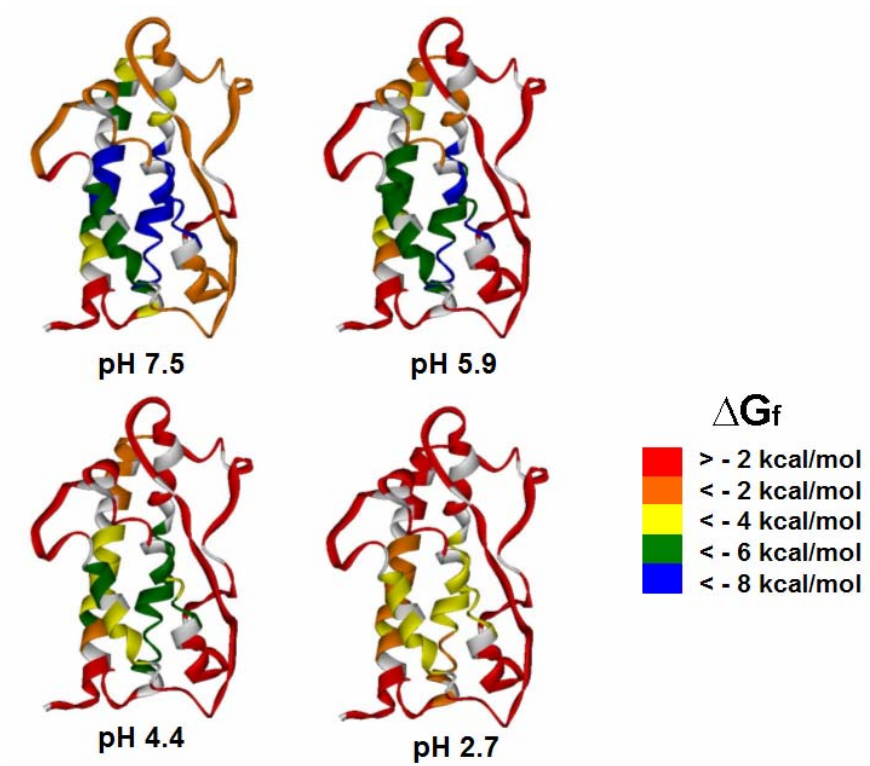
A leading biogenerics manufacturer had ExSAR compare their follow-on biologic to a product already marketed by a large pharmaceutical company. The client's intent was to strengthen the claim that the branded and follow-on products were structurally identical. As illustrated in Figure 4, this was in fact what we determined. For instance the client's follow-on product exhibited a nearly identical amide hydrogen exchange rate behavior as the branded product as well as API and USP "gold" standards. We also discovered that both the follow-on and branded products were similarly less dynamic in an area corresponding to residues 12 through 28 when formulated in one of the two commercially available conditions (formulation "B").



**Figure 4.** The result of a six-way H/D-Ex comparison consisting of: (a) a United States Pharmacopeia (USP) standard in formulation-A, (b) manufacturer’s active pharmaceutical ingredient (API) in formulation-A, (c) manufacturer’s branded product in formulation-A, (d) client’s follow-on product in formulation-B, (e) manufacturer’s branded product in formulation-B and (f) client’s follow-on product in formulation-B. Each horizontal colored block represents an analyzed peptic peptide. Numbering is indicative of the relative position in the protein sequence. The deuteriation level of each peptide is color-coded as shown for each of the five time points (30, 100, 300, 1,000 and 3,000 s).

### Case study #3 –Sensor of Structural Stability

Hydrogen deuterium exchange is a thermodynamic sensor of structural stability. The Gibbs free energy of folding ( $\Delta G_f$ ) can be calculated from the rate of exchange. The more negative the  $\Delta G_f$  the more stable the structure. To better illustrate this application, we deliberately disrupted the stability of human Growth Hormone (hGH) by exposing the protein to buffers of increasing acidity. Next we measured the rate of exchange of approximately 30 sequence segments spanning the full length of hGH. For each of these regions we calculated  $\Delta G_f$ . The magnitude of  $\Delta G_f$  was then assigned a color going from red- not very stable, to blue- highly stable. As illustrated below in Figure 5, we colored the 3-dimensional structure of hGH according to the free energy of folding value of each segment. Observe the blue to yellow transition in the “core” region of the structure as the buffer goes from pH 7.5 to 2.7. This dramatic change in stability corresponds to an approximate 4 kcal/mol loss in stability in the “core” region of the protein ( $\Delta G_{pH\ 7.5} - \Delta G_{pH\ 2.7} = \Delta\Delta G_f = -4$  kcal/mol).



**Figure 5.** H/D-ex determined Gibbs free energy of folding ( $\Delta G_f$ ) illustrated on the X-ray structure (PDB identifier 1HGU) of human Growth Hormone (hGH).

### III. References

1. Herrera S. Biogenerics standoff. Nat Biotechnol 2004;22:1343-1346.
2. Editorial. Overdue process. Nat Biotechnol 2004;22:1329.

### **III. ExSAR Competencies/Deliverables**

ExSAR has offered H/D-Ex services since 2002. ExSAR has conducted over a dozen antibody based epitope mapping studies for major pharmaceutical and biopharmaceutical clients. It offers a collaborative and consultative approach, with verbal and written presentation of results. Its scientific leadership includes Dr. Charles Cantor, Dr. William DeGrado, and Dr. S. Walter Englander, each members of the National Academy of Science. Projects are conducted under the supervision of Dr. Yoshitomo Hamuro.

#### **William F. DeGrado, Ph.D. - Chairman of the ExSAR Scientific Advisory Board**

Dr. DeGrado is the George W. Raiziss Professor of Biochemistry and Biophysics at the University of Pennsylvania and he is a member of the National Academy of Sciences. His published research includes contributions to the fields of protein design, synthesis of peptidomimetics, and characterization of membrane-active peptides.

#### **S. Walter Englander, Ph.D. – Member, ExSAR Scientific Advisory Board**

Dr. Englander is the Jacob Gershon-Cohen Professor of Medical Science and Professor of Biochemistry and Biophysics at the University of Pennsylvania. He is a member of the National Academy of Sciences, Honorary Fellow of the Biophysical Society and Honorary Fellow of the American Association for the Advancement of Science. His work has focused on internal protein motions and correlations to amide hydrogen exchange rates.

#### **Charles R. Cantor, Ph.D. - Director, ExSAR Board of Directors**

Dr. Cantor is the Chief Scientific Officer and Chairman of Sequenom, and a member of the National Academy of Sciences. He was previously the chair and professor of the department of biomedical engineering and biophysics, and director of the Center for Advanced Biotechnology, at Boston University. Prior to this, Dr. Cantor held faculty positions at Columbia University. He was also director of the Human Genome Center Project of the Department of Energy at Lawrence Berkeley Laboratory. Dr. Cantor has published more than 325 peer reviewed articles and has been granted 26 U.S. patents.

#### **Yoshitomo Hamuro, Ph.D.—Senior Director of Technology Development**

Dr. Hamuro has led the development of hydrogen/deuterium exchange mass spectrometry analysis of protein dynamics, protein-ligand interactions and protein-protein interactions at ExSAR since joining in 2002. Prior to joining ExSAR, he was instrumental in the development of modern H/D-Ex technology at the University of California, San Diego, in the laboratory of Professor Virgil Woods. Dr. Hamuro conducted postdoctoral research on combinatorial chemistry and solid-phase chemistry at DuPont and later on antibacterial  $\beta$ -peptides at the University of Pennsylvania under Professor William DeGrado. He obtained his Ph.D. in 1996 from the University of Pittsburgh on protein structure mimetics.