# A new method for monitoring the integrity of humanized monoclonal antibodies using surface plasmon resonance

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# A new method for monitoring the integrity of humanized monoclonal antibodies using surface plasmon resonance

**In this application note, a novel method for the integrity testing of humanized monoclonal antibodies using surface plasmon resonance (SPR) is presented. A panel of binding-mode sensitive reagents (BMSRs) covering the several domains of IgG1 and capable of recognizing stress-related changes in five humanized monoclonal antibodies, was selected through a screening procedure. The specificity and selectivity of these reagents were tested by comparing their binding behavior to several forms of monoclonal antibody X and Y (mAbX and mAbY), wherein significant differences in binding patterns were found between the non-stressed and stressed variants and dimers. The BMSR binding site and altered binding properties could be connected to stress-induced changes at the molecular level, as revealed by spectrometric, chromatographic, and electrophoretic methods. Using a new software-based statistical evaluation strategy, the detection limit of oxidized methionine, deamidated aspargine, and dimer content ranged from 0.4% to 4%, depending on the nature of modification and BMSR used.**

## Introduction

Biacore™ software supports determination of rate and affinity constants when the binding mode is known. However, in some cases, for instance when the interaction is heterogeneous or of an unknown type, or the off-rate is too slow, the evaluation of data is difficult. This problem can be overcome with a new approach to sensorgram analysis, which involves statistical comparison of the shape of sample sensorgrams with that of a reference standard. The standard is derived from at least two (preferably more) replicate runs with the same material, and represents the range of acceptable experimental variation between equivalent samples.

Comparison is performed on the numerical sensorgram data without reference to the kinetics or other properties of the interaction. Sensorgram comparison is primarily useful in comparing the behavior of samples that are expected to be the same, such as studies of resemblance, stability, and batch-to-batch variation, where detailed kinetic or affinity analysis is difficult or not appropriate. The information obtained gives numerical values for the extent of similarity between sample and reference standard but does not provide any further characterization of observed differences.

Here, we show how this sensorgram comparison was used for a selection of reagents sensitive to stress-induced changes for the binding behavior to IgG1 and in an application testing the integrity of humanized monoclonal antibodies using these reagents.

## Selection of reagents recognizing stress-related changes

Five humanized monoclonal antibodies were exposed to two types of forced degradation: elevated pH (pH 9.0 for three days at 37°C) and oxidation (pH 7.0 in the presence of 0.3% of hydrogen peroxide for 12 h at room temperature). The stressed and non-stressed antibodies were then screened against ~30 reagents and tested for differences in binding characteristics. Six reagents*, denoted as BMSR 1 to BMSR 6, binding to different domains of IgG1 (Fig 1) showed altered interaction patterns with all the humanized monoclonal antibodies tested. These reagents were thus selected to further explore their sensitivity to stress-related changes and dimerization of the antibodies tested.

* Supplementary information on BMSR1 to BMSR6 is available in the Optimal Interactions Club at www.gelifesciences.com/DiPIAdigital.

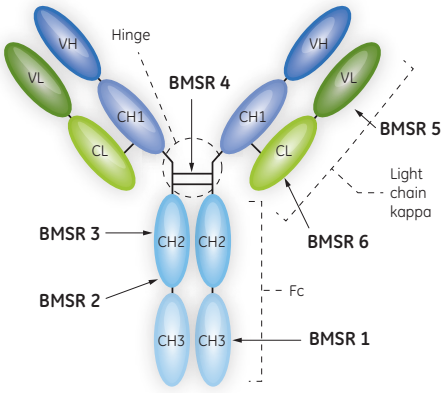


**Fig 1.** BMSRs map the surface of IgG1 by binding to epitopes in different domains.

## Forced degradation of mAbX detected by BMSRs

The applicability of selected reagents for the analysis of integrity was examined on monoclonal humanized antibody X (mAbX) of IgG1 subtype that was exposed to three types of forced degradation: oxidation, pH stress, and light stress (Fig 2). To investigate stress-caused modifications on a molecular level, the mAbX variants were analyzed by size-exclusion chromatography–multi-angle light scattering (SEC-MALS), mass spectrometry (MS) (only forced-oxidized and pH-stressed), and ion exchange chromatography (IEC) (only light-stressed) (Table 1). All mAbX variants as well as SEC-MALS, MS, and IEC data were kindly provided by F. Hoffmann-La Roche Ltd., Basel, Switzerland.

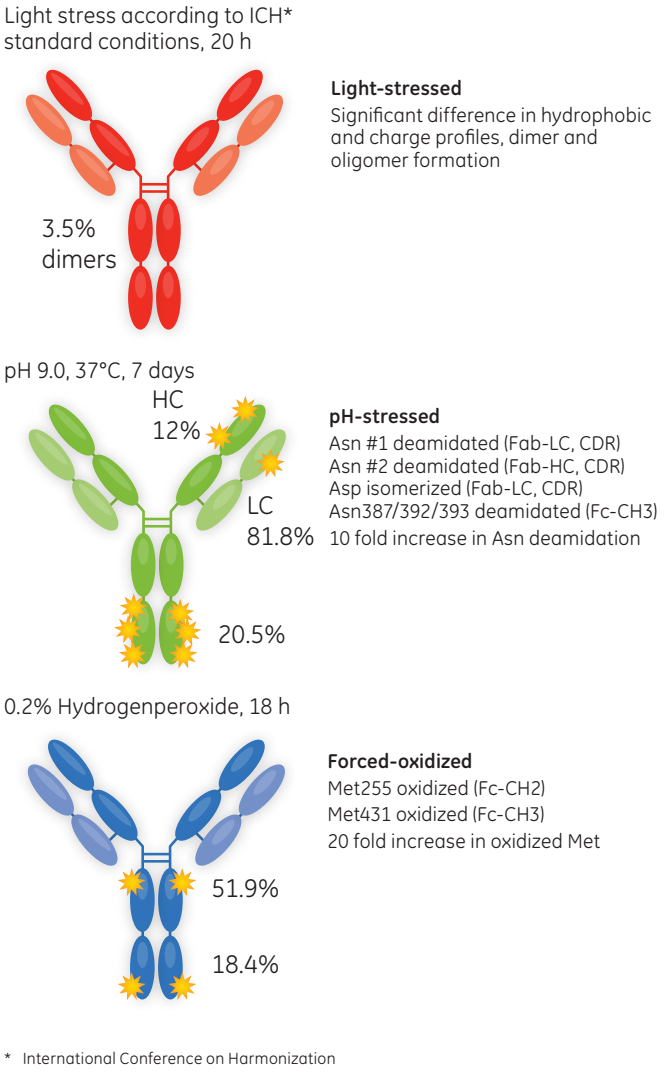


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**Fig 2.** The stress scheme of mAbX. Stress type and procedure, affected amino acids, and degree of modification are indicated.

**Table 1.** Results of analyses by SEC-MALS, MS, and IEC performed on mAbX variants at F. Hoffmann-La Roche Ltd., Basel, Switzerland

| Technology | Forced-oxidized mAbX | | | | pH-stressed mAbX | | | | Light-stressed mAbX | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***SEC-MALS*** | | | | | | | | | | | | |
| Form | Monomers | Dimers | Fragments | Oligomers | Monomers | Dimers | Fragments | Oligomers | Monomers | Dimers | Fragments | Oligomers |
| Non-stressed, area (%) | 99.9 | 0.1 | 0.0 | 0.0 | 99.9 | 0.1 | 0.0 | 0.0 | 99.9 | 0.1 | 0.0 | 0.0 |
| Stressed, area (%) | 99.8 | 0.2 | 0.0 | 0.0 | 99.3 | 0.6 | 0.2 | 0.0 | 96.1 | 3.5 | 0.2 | 0.2 |
| ***MS*** | | | | | | | | | | | | |
| Amino acids | Met255 oxidized | | Met431 oxidized | | Asn#1 deamidated | Asn#2 deamidated | Asp isomerized | Asn387/ 392/393 deamidated* | N.D. | | | |
| Location | Fc-CH2 | | Fc-CH3 | | Fab-LC, CDR | Fab-HC, CDR | Fab-HC, CDR | Fc-CH3 | | | | |
| Content in non-stressed (%) | 3.5 | | 1.4 | | 8.1 | 1.0 | 5.0 | 1.2 | | | | |
| Content in stressed (%) | 51.9 | | 18.4 | | 81.8 | 12.0 | 11.3 | 20.5 | | | | |
| ***IEC*** | | | | | | | | | | | | |
| | N.D. | | | | N.D. | | | | Significant difference in hydrophobic behavior and charge profile, dimers, and oligomers formation | | | |

* Modification of all three amino acids in penny motif integrated as a sum.
N.D. = not determined

In the SPR analysis, BMSRs were immobilized on a Series S Sensor Chip CM5 in a Biacore T200 instrument using a standard amine coupling procedure to a level that gave a 10 to 100 RU response 3 min after starting the injection of 100 nM antibody. After 10 min of antibody dissociation, the immobilized surface was regenerated using reagent-specific regeneration solution (see www.gelifesciences.com/DiPIAdigital). Each mAbX variant was analyzed three times against each BMSR.

Distinct differences in binding pattern to forced-oxidized, pH-stressed, and light stressed variants could be observed when compared to non-stressed mAbX (Fig 3).

BMSR 1, BMSR 2, BMSR 3, and BMSR 4 binding to $C_H2$ and $C_H3$ domains (containing oxidated methionines) and to the hinge region were sensitive to forced oxidation, showing an altered stability pattern expressed by changed dissociation. For BMSR 1, BMSR 2, and BMSR 4, faster dissociations were observed meaning less stable binding to oxidized antibody, whereas BMSR 3, which binds to the $C_H2$ domain, showed a slower dissociation rate, indicating more stable binding of this reagent. Similarly, BMSR 1, BMSR 5, and BMSR 6 were detecting modification in the $C_H3$ domain and light chain of the pH-stressed variant by showing more stable binding, while BMSR 1 and BMSR 2 were dissociating faster from the light-stressed variant, revealing some changes in $C_H2$ and $C_H3$ domains (Fig 3).

Interestingly, BMSR 1 and BMSR 2 were capable of identifying changes related to several stress variants whereas BMSR 3 was specific for oxidation and BMSR 5 and BMSR 6 for deamidation in the CDR region.

Additionally, 2-Dimensional Differential Gel Electrophoresis (2D-DIGE) (Fig 4A) was used to examine changes in size and charge patterns of stressed variants in relation to wild type mAbX. Considerable differences could be observed in the size and charge pattern for pH-stressed and light-stressed variants (Figs 4B and 4C), whereas no deviations were detected for the forced-oxidized mAbX form.

In pH-stressed mAbX, a distinct shift of isoelectric point of light chain, from 7.8 to 7.0, depending on deamidation of Asn #1 that increases negative charge, was observed. Other pI shifts of heavy chain were seen as a train of spots with different charge, most likely due to deamidations of several aspargines in this region. Covalently linked dimers with various charge were also detected (Fig 4B). In the light-stressed variant, an increase of the dimers and oligomers content was seen (Fig 4C).

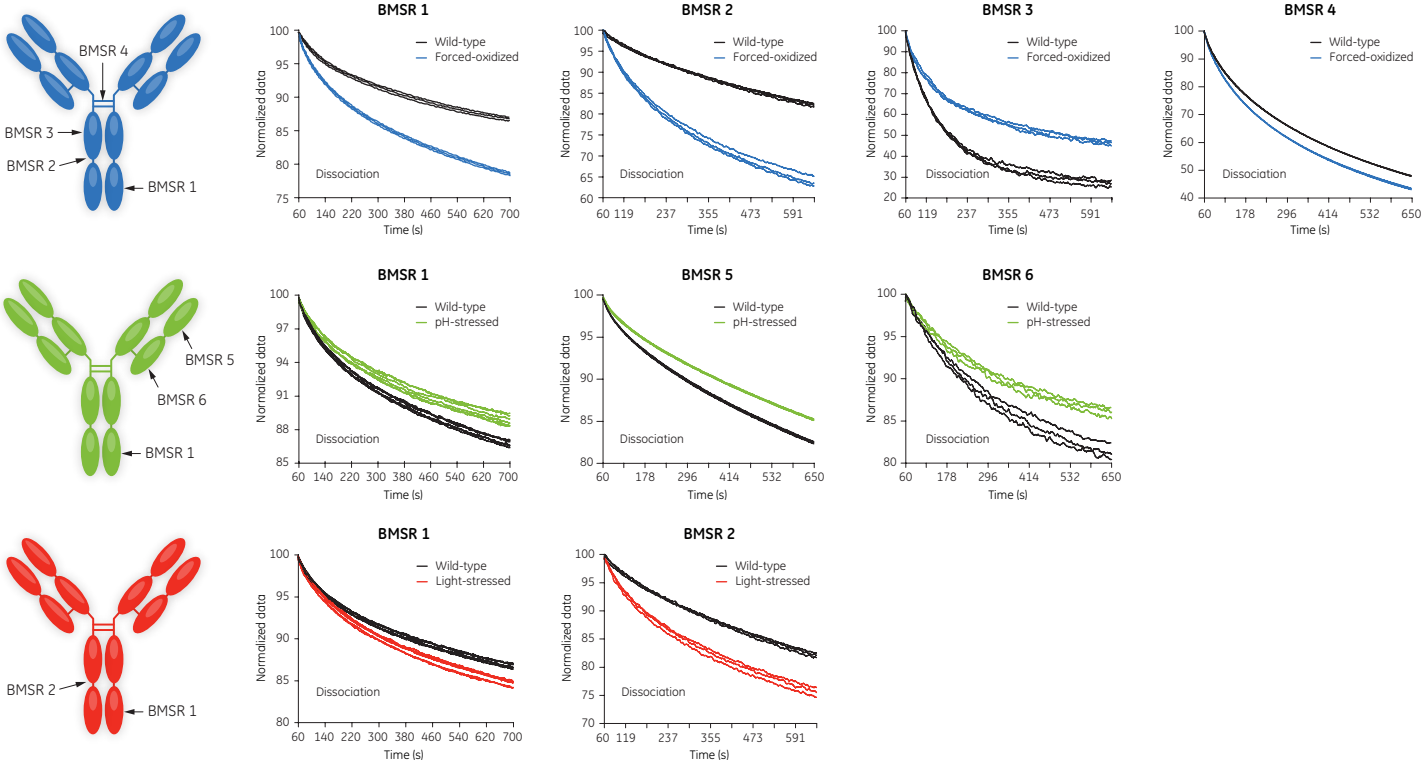


**Fig 3.** Differences observed in binding stability pattern to the reagents that were sensitive to stress-related changes in forced-oxidized, pH-stressed, and light-stressed mAbX variants.

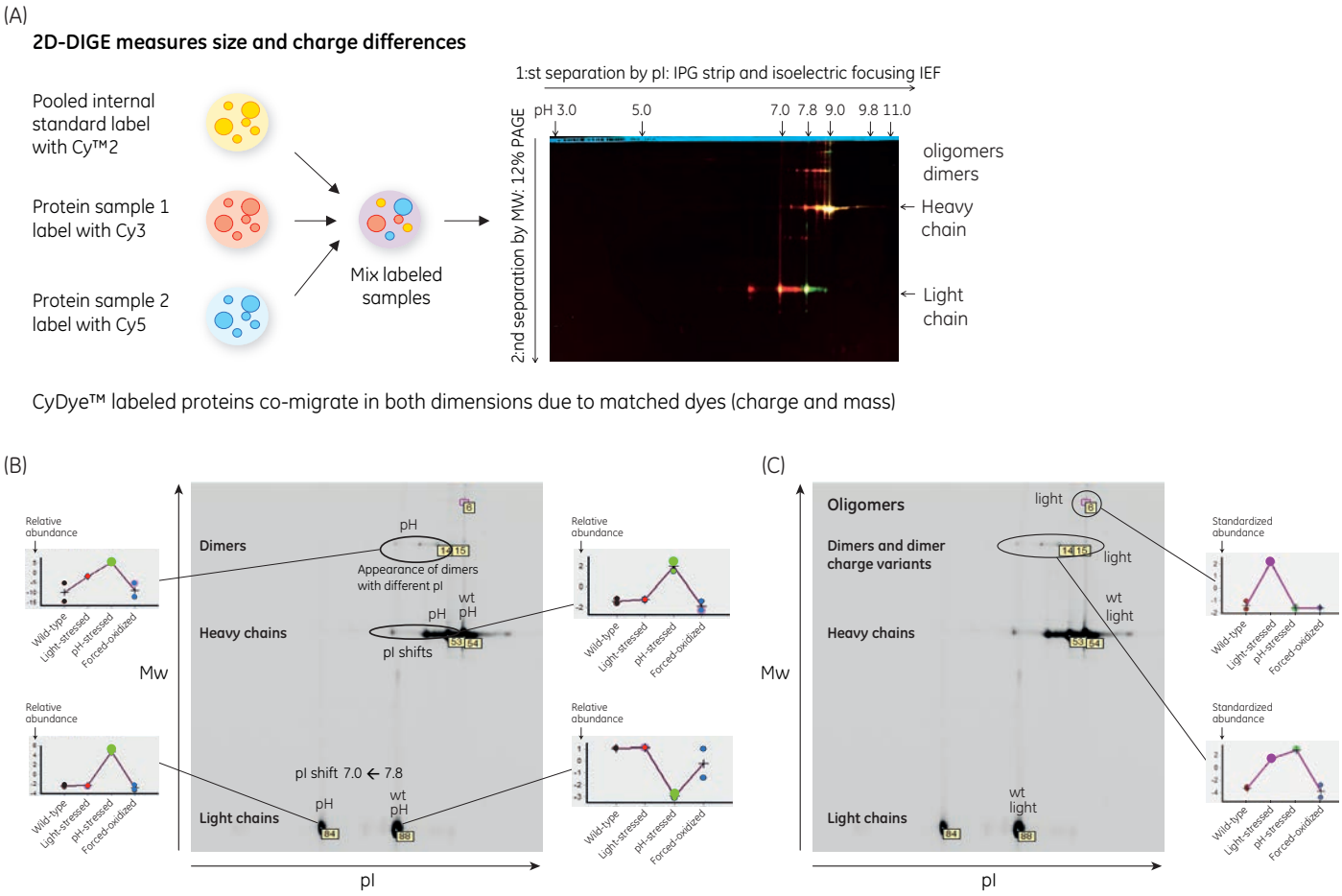


**Fig 4.** An overview of 2D-DIGE procedure (A) and results (gels) obtained for pH-stressed (B) and light-stressed (C) variants together with plots showing relative abundance of each protein against the normalized internal standard. Abbreviations used: wt = non-stressed wild-type mAbX, pH = pH-stressed mAbX, light = light-stressed mAbX.

Through spectrophotometric and chromatographic analyses (Table 1) as well as from 2D-DIGE experiments, the information about stress-affected amino acids, their degree of modification, and charge/aggregation/fragmentation pattern was obtained and the stress-induced differences at molecular level could be easily connected to the changes detected in the binding behavior of the reagents. An agreement could be observed between location of the affected amino acids and the binding site of this reagent that demonstrated altered binding behavior.

## A statistical approach to sensorgram comparison

To assess the sensitivity of the reagent for detection of stress, a novel statistical approach to data evaluation was used to compare sensorgrams of non-stressed antibody, used here as standards, with sensorgrams of stressed antibody variants. All sensorgrams were derived from multiple runs of 100 nM variants under the same conditions to establish the extent of experimental variation. The sensorgram comparison procedure (Fig 5) involves:

**(i)** The normalization of replicate sensorgrams for standard and all variants to be compared to standard, to the same scale between 0 and 100, in order to focus on the sensorgram shape.

**(ii)** Calculation of the average and two standard deviation (SD) curves (here: ± 3 SD) for standard replicates.

**(iii)** Calculation of the percentage of data points in a sample curve that falls within the selected SD borders.

**(iv)** Calculation of similarity rating values based on the sample points that fall outside the SD borders.

**(v)** Determination of the similarity score for each sample from the percentage of points inside and outside the SD borders and from the similarity rating, according to the equations below.

$$\text{Similarity rating} = \frac{\Sigma(\text{standard distance})^2}{\Sigma(\text{sample distance})^2}$$

$$\text{Similarity score} = (\%\ \text{points inside} \times 1) + (\%\ \text{points outside} \times \text{similarity rating})$$

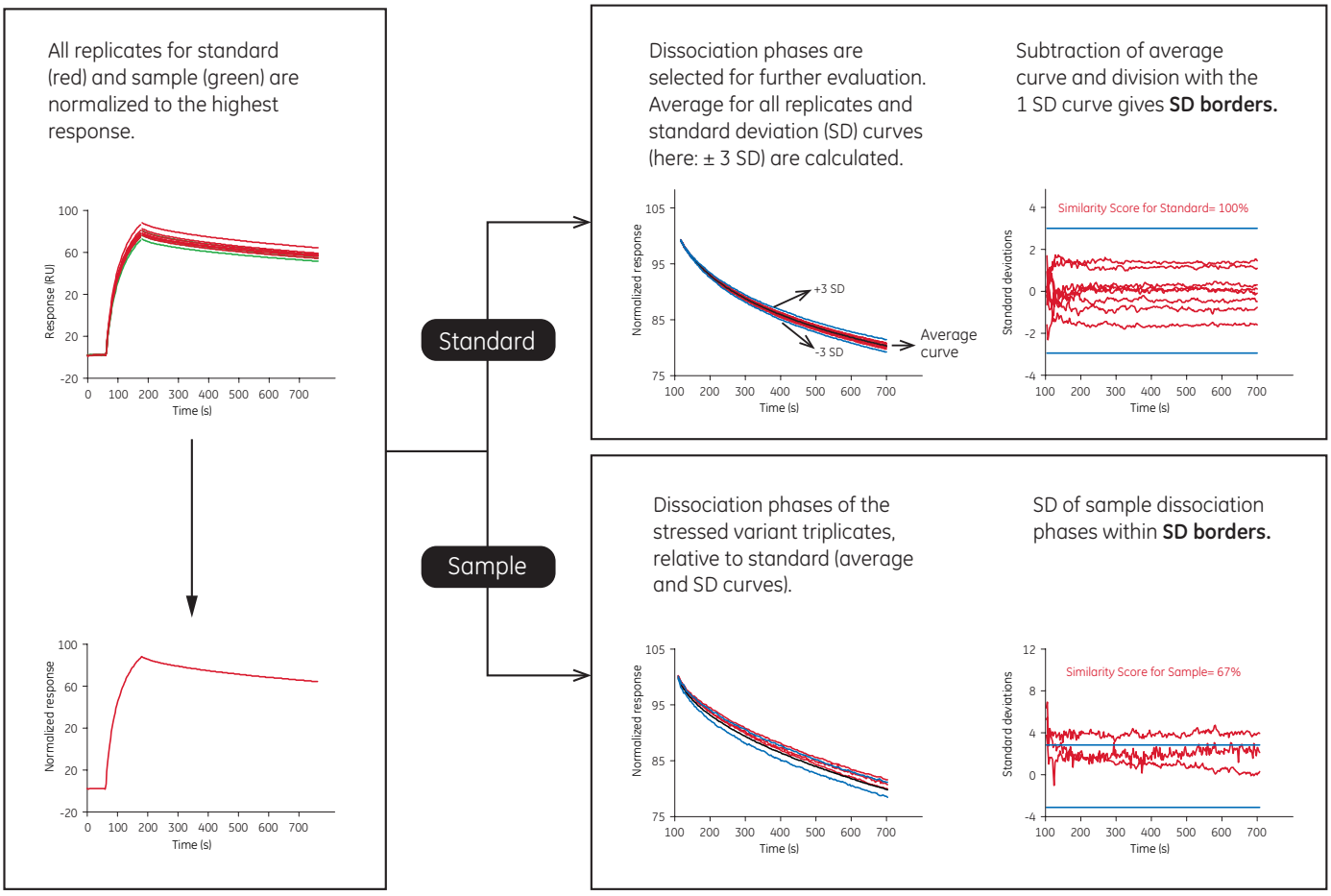


**Fig 5.** Overview of evaluation procedure based on sensorgram comparison.

## Sensitivity of BMSRs for detection of stress-related modifications

Controlled spike-in experiments were performed to evaluate the sensitivity of the detection of stress-related changes by the BMSRs. In these experiments, the forced-oxidized and pH-stressed mAbX variants, for which identity and degree of degradation of affected amino acids were well-defined, were examined.

Two components solutions were prepared containing non-stressed and stressed mAbX (forced-oxidized or pH-stressed), in which the content of the latter was varied from 0% to 100% with a 10% interval, while maintaining the total concentration of antibody at 100 nM. At the amino acid level, in mixtures spiked with forced-oxidized variant, the content of oxidized Met 255 and Met 431 varied from 5.2% to 52% and from 1.8% to 18.4%, respectively. Similarly, in the pH-stressed spike-in solutions, the content of deamidated Asn#1 and Ans#2 in the CDR region, and of deamidated penny motif in the $C_H3$ domain, varied from 8.2% to 82%, from 1.2% to 12%, and from 2% to 20.5% respectively.

In addition, the ability of BMSRs to detect dimers was studied using mAbY monomer and dimer (kindly provided by F. Hoffmann-La Roche Ltd., Basel, Switzerland).

Two components solutions containing monomer and dimer of mAbY consisted of a 2-fold dilution series of dimer (0.7, 1.5, 3, 6, 12, 25, 50, 100%), in the monomer solution, with the final concentration of each spiked mixture being 100 nM.

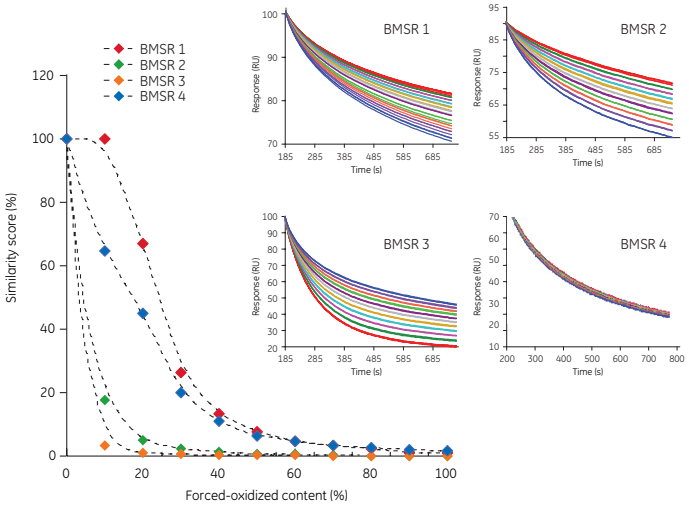


**Fig 6.** Correlation plot showing dependence between the similarity score and the content of forced-oxidized variant for four BMSRs. Corresponding dissociation phases for spiked solutions containing 0% to 100 % of forced-oxidized variant, are shown to the right.

In the binding analysis to BMSRs, the non-stressed mAbX variant and mAbY monomer were tested six to eight times and used as standards while each spiked mixture of stressed variants and dimer were examined three times. Altered sensorgram parts (i.e., the dissociation phases of all mAbX variants and the whole sensorgram of mAbY monomer and all spiked dimer variants) were used to calculate the similarity score values (average from replicates) in the prototype software of Biacore T200 version 3.0, using the *Sensorgram Comparison* functionality, as described in Figure 5.

The sensitivity of detection of changes in stressed mAbX, assigned to modifications at the amino acid level and dimerization, varied with BMSR and type of stress.

Assuming that a similarity score of 80% corresponds to the detection limit, the quantity of detectable stress-modified variant could be calculated from the correlation plots (Figs 6, 7, and 8) and translated to the percentage of modified amino acids (Table 1) or the dimer content that could be distinguished by the BMSRs.

Approximately 0.4% to 3% of oxidized methionines positioned in $C_H2$ and $C_H3$ domains were detected in forced-oxidized mAbX by four BMSRs binding to the same regions. The sensitivity of detection of deamidated aspargines in the penny motif of the pH-stressed variant by BMSR 1 was approximately 3%. BMSR 5 and BMSR 6, binding to the κ-light chain, were able to detect modifications of two aspargines located in the CDR region, with sensitivity ranging from 3% to 20% of affected amino acid (Table 2).

BMSR 1 appears to be most sensitive in recognizing the presence of dimer, detecting 4% of this mAbY form, whereas only 30% of dimer was revealed by BMSR 3. BMSRs 2, 4, 5, and 6 detected from 7 to 12% of dimer (Table 2).

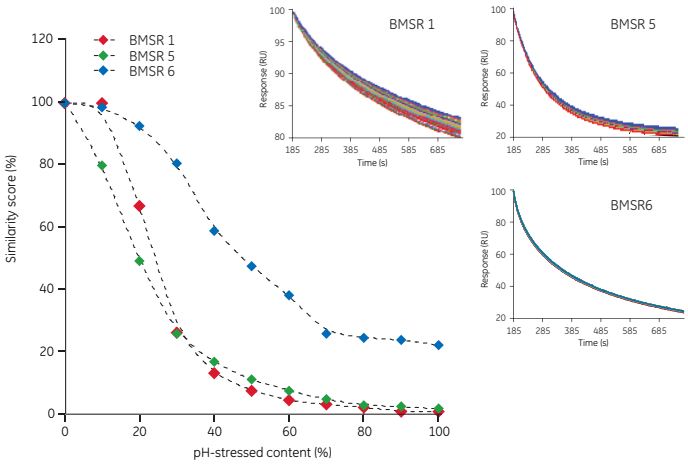


**Fig 7.** Correlation plot showing dependence between similarity score and the content of pH-stressed variant for three BMSRs. Corresponding dissociation phases for spiked solutions, containing 0% to 100 % of pH-stressed variant, are shown to the right.

**Table 2.** The limit of detection of stressed mAbX variants and mAbY dimer calculated for each BMSR and, for pH-stressed and forced-degraded forms, translated into percentage of detected amino acids located closest to the BMSR binding site

| Spiked variant | Reagent | Closest affected amino acid | Limit of detection corresponding to 80% similarity score (%) | Detected amino acid (%)* |
|---|---|---|---|---|
| Forced-oxidized mAbX | BMSR1 | Oxidized Met431 | 15 | 3 |
| | BMSR2 | Oxidized Met431/Met255 | 2 | 0.4/1 |
| | BMSR3 | Oxidized Met255 | 2 | 1 |
| | BMSR4 | Oxidized Met255 | 5 | 3 |
| pH-stressed mAbX | BMSR1 | Deamidated Asn387/392/393 | 15 | 3 |
| | BMSR5 | Deamidated Asn#1/Asn#2 | 10 | 8/1 |
| | BMSR6 | Deamidated Asn#1/Asn#2 | 25 | 20/3 |
| Dimer mAbY | BMSR1 | N/A | 4 | N/A |
| | BMSR2 | N/A | 12 | N/A |
| | BMSR3 | N/A | 30 | N/A |
| | BMSR4 | N/A | 7 | N/A |
| | BMSR5 | N/A | 10 | N/A |
| | BMSR6 | N/A | 10 | N/A |

* Percent of detected amino acid based on MS data in Table 1.

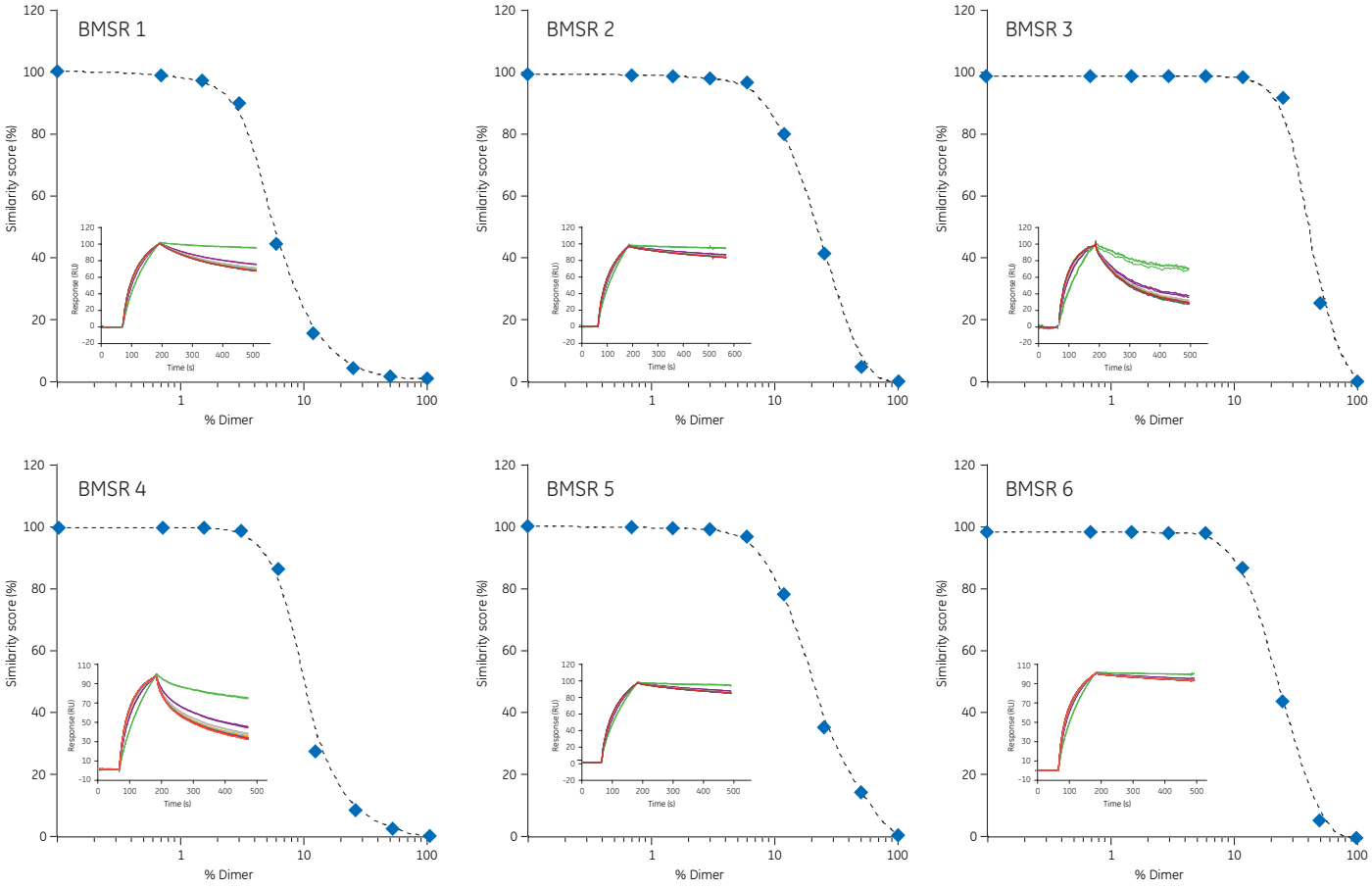


**Fig 8.** Correlation plots showing dependence between similarity score and dimer content for each BMSR. Insets show corresponding normalized sensorgrams of binding of monomer, and dimer-spiked solutions, containing 0.7% to 100% of dimer, to BMSRs. The similarity score was calculated using the monomer binding curves (shown in red) as standard. The sensorgrams of 100% dimer are shown in green.

## Conclusions

An array of BMSRs could be selected and used to rapidly reveal stress-induced modifications in an antibody by SPR. The sensitivity of detection varied with stress type, affected amino acid, distance to this amino acid from the BMSR binding site, and BMSR used. The oxidized methionines were detected with the highest sensitivity between 0.4% and 3% of stress-modified amino acid, by four BMSRs binding in a close neighborhood to the oxidation sites. 3% of deamidated aspargines located in the $C_H3$ region of pH-stressed antibody could be detected by a reagent binding to this region. Although CDR-binding probes were not available, it was possible to detect deamidation of between 1% and 20% of aspargines in this part using two reagents binding to the κ light chain. The presence of dimers was detected with considerably lower sensitivity revealing no more than 4% of dimer using BMSR 1 as the $C_H3$-binding reagent.

This application note demonstrates the potential of a label-free binding assay for integrity testing of antibodies using BMSRs and describes a new sensorgram comparison technique that allows an objective evaluation of similarity between molecules. In the future, the development of more numerous BMSRs with well-defined binding sites densely covering an antibody can provide a fingerprint of this molecule, thus making any changes more easily identifiable.


## *Acknowledgements*

We thank Dr. Hermann Beck at F. Hoffmann-La Roche Ltd., Basel, Switzerland, for fruitful discussions and for providing mAbX and mAbY variants and analytical data shown in Table 1.


## Ordering information

| Product | Code number |
|---|---|
| Biacore T200 Processing Unit | 28975001 |
| Series S Sensor Chip CM5, pack of 3 | BR100530 |
| Amine Coupling Kit | BR100050 |
| Immobilization buffer, 10 mM sodium acetate pH 4.0 | BR100349 |
| Immobilization buffer, 10 mM sodium acetate pH 4.5 | BR100350 |
| Immobilization buffer, 10 mM sodium acetate pH 5.0 | BR100351 |
| 10 mM glycine-HCl pH 1.5 | BR100354 |
| 10 mM glycine-HCl pH 2.0 | BR100355 |
| 10 mM glycine-HCl pH 2.5 | BR100356 |

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