FIELD APPLICATION REPORT

Multimode Detection

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A Plate-based Time-resolved Fluorescence Method for Quantifying Carbonylation Damage in Protein Samples

Introduction

The Baltic Sea is a large brackish water in Northern Europe surrounded by nine countries. The natural characteristics of the Baltic Sea make it especially vulnerable to both human impact and natural changes. Animals and plants inhabiting the Baltic Sea are subjected to multiple stressors, from considerable spatial, seasonal and vertical changes in hydrography, to human generated harmful substances and nutrient loads (HELCOM, 2011). In addition, the Baltic Sea has been subject to greater than the reported global mean temperature increase over the past century (The BACC Author Team, 2008). Monitoring and studying the effects of various environmental stressors on Baltic Sea flora and fauna is challenging. In this application note we show how the application of high sample density microplate reader technology combined with high sensitivity, time-resolved fluorescence (TRF) measurement, contribute to research in this important field by allowing the measurement of some of the indicators of oxidative stress in a faster, simpler and higher throughput way compared to previously used methods.

Biological background

Reactive oxygen species (ROS) such as free radicals and peroxides are capable of modifying DNA, lipids and proteins. Oxidative stress is a state where the level of ROS exceeds the organism's capacity to mitigate the damage by detoxifying the reactive intermediates and/or by repairing the resulting damage to cellular macromolecules. Cells' inability to defend themselves against oxidative damage to their DNA, lipids and proteins can ultimately lead to impaired cellular function and cell death. A variety of diseases are linked to oxidative stress including age-related neurodegenerative diseases, cardiovascular diseases, cancer and type II diabetes (Halliwell & Gutteridge, 2007; Monaghan et al, 2009). Oxidative stress is also an important

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component of the stress response in wild animals exposed to a variety of environmental changes (e.g. Isaksson 2010; Lesser, 2006).

Oxidative stress can be studied by measuring any of the four components of redox status: (1) free radicals, (2) antioxidant defense, (3) oxidative damage, and (4) repair mechanisms. A multifaceted approach is often necessary to assess oxidative stress and to understand its consequences for the organism. (Halliwell & Gutteridge, 2007; Monaghan et al, 2009).There is a wide range of methods to study any of the four components of oxidative stress. A comprehensive battery of methods to cover all the different components would potentially require access to diverse instrumentation, substantial sample volumes, reagents and laboratory time. Plate reader technologies offer a variety of accessible methods especially suitable for screening larger numbers of samples which is important because of the need to test multiple specimens (typically 20 to 50) in order to adequately represent biological variation in a study population. They also allow testing in lower sample volumes, thus reducing the volume of biological material needed from each specimen. In this paper we present a new easy-to-use method to assess oxidative damage by quantifying carbonyl modification in protein samples. The goal in developing this method was to enable more efficient and faster screening of protein carbonylation damage in large biological sample sets by reducing and simplifying the steps needed to conduct the assay.

Carbonylation is one of the most widely used biomarkers of oxidative damage to proteins. Carbonylation is a modification to proteins where a functional group C=O is incorporated into an amino acid side chain. Carbonyl modifications to proteins are irreversible and irreparable; cells need to remove the affected proteins by proteolysis. In oxidative stress the level of oxidative damage exceeds the cells' ability to degrade carbonylated proteins. Highly carbonylated proteins form proteolysis-resistant high-molecular-weight aggregates that accumulate into cells over time. Such aggregates can affect cell function and become cytotoxic. Cells can dilute aggregate load with each cell division, but permanent cells such as neurons are especially vulnerable to protein aggregate accumulation and carbonylated aggregates are associated with a number of progressive age-related neurological disorders such as Alzheimer's disease and Parkinson's disease. (Grimsrud et al, 2008; Nystrom, 2005; Yan & Forster, 2011).

Assay principle

Carbonyl groups themselves do not have spectrophotometric characteristics and so specific chemical probes are needed to detect and quantify carbonylation. N'-aminooxymethylcarbonylhydrazino d-biotin, also known as Aldehyde Reactive Probe (ARP), was first introduced for detecting protein carbonyls in 2006 (Chavez et al, 2006; Chung et al, 2008). Before that, ARP was already widely used for detecting abasic sites (aldehyde/keto groups) in DNA strands. ARP is a biotin probe that forms a chemically stable oxime derivative with the aldehyde/keto group in carbonylated proteins. Because ARP recognises carbonylated sites in both nucleic acid strands and amino acid chains, nucleic acids may interfere with the results if they are not removed from the samples.

In this experiment, ARP was conjugated with proteins and bound to the plate following which europium-labeled streptavidin was added, which binds via a strong non-covalent affinity bond to the biotin-tag of ARP.

The DELFIA® method used in these experiments is built upon time-resolved fluorescent technology, made possible by the unique fluorescent properties of lanthanides. The amplified fluorescent signal is detected using time-resolved fluorometry; the intensity of the fluorescent signal is proportional to the amount of analyte present in the sample. This particular TRF method offers a number of advantages for this research over the ELISA and colorimetric methods commonly used. Firstly, higher sensitivity means that even lower sample volumes can deliver the desired signal level. Secondly, the lower number of preparation steps in the assay and the faster measurement times lead to faster results which can translate into earlier research outcomes.

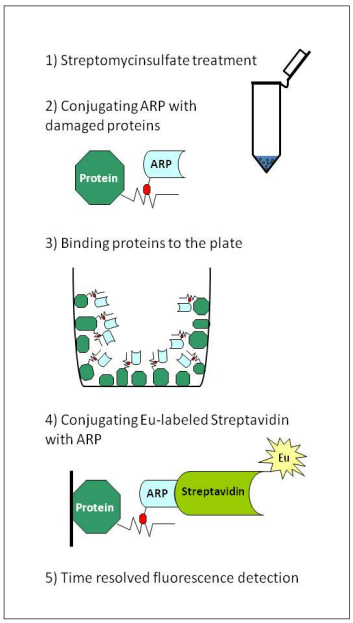

Figure 1. Assay principle

Materials and Methods

BSA standard preparation

Standard curves were constructed by mixing varying proportions of reduced and oxidized bovine serum albumin (BSA). Reduced and oxidized BSA was prepared according to Alamdari et al. 2005. Carbonyl contents of BSAs were measured with a colorimetric assay (Alamdari et al, 2005) on the EnSpire® Multimode Plate Reader. The reduced BSA's carbonyl content was 0 nmol/mg and oxidized BSA's 2.7 nmol/mg.

Sample preparation

The protein contents of the biological samples and standards were determined using the EnVision® Multilabel Plate Reader with the Bradford method using a BioRad Protein Assay (BioRad, Espoo, Finland) with BSA (Sigma Chemicals, St. Louis, Missouri, USA) as the standard. The samples and standards were then diluted with 0.1 M K2HPO4 + 0.15 M KCl-buffer (pH 7.4) to a protein concentration of 4 mg/ml. 20 % streptomycin sulfate (in 50 mM potassium phosphate, pH 7.2) was added 1:20 to precipitate the nucleic acids in the biological samples. After 15 min incubation at room temperature the extracts were clarified by centrifugation (10 min, +4 ℃, 6000 g). Five µl of sample supernatant or standard was then incubated 45 minutes with 15 µl of 10 mM ARP at room temperature after which 5 µl of ARP treated sample/standard was diluted with 995 µl 0.1 M K2HPO4 + 0.15 M KCl-buffer (pH 7.4) in a new Eppendorf tube (step 1 Figure 1).

Time-resolved fluorescence (TRF) assay

Binding proteins to plate

The assay was performed in DELFIA Yellow plates (product number AAAND-0001), that have a high protein binding capacity combined with exceptionally low fluorescence background. 200 µl of the diluted standard or biological samples (5 µg protein in 1 ml buffer), and buffer without protein (blank) were added into the DELFIA Yellow plates' wells in triplicate. The plate was incubated for one hour at 37 ℃. Initially, three different incubation setups were tested, of which one hour at 37 ℃ and over night at +4 ℃ gave similar results. After the incubation, the plate was washed twice with DELFIA wash buffer (PerkinElmer) using DELFIA Platewash (PerkinElmer) (step 2 Figure 1).

Streptavidin

Europium-streptavidin label (PerkinElmer) was prepared according to the manufacturer's instructions. 200 µl of the label was added to the plate wells, and the plate was incubated for one hour at room temperature on a plate shaker (slow mode). After incubation, the plate was washed with DELFIA wash buffer four times using the plate washer. 200 µl of the DELFIA enhancement solution (PerkinElmer) was added to the wells, the plate incubated five minutes at room temperature on a plate shaker (slow mode) and TRF measured with EnVision and EnSpire plate readers[1] (step 3 Figure 1).

	EnSpire (monochromator based)	EnVision (Filter based)
Excitation	320nm	340nm
Emission	615nm	615nm
Delay time	400µs	400µs
Window width	400µs	400µs
Number of flashes	750	100
Measurement height	7.5mm	6.5mm

Table 1. Instrument settings

Results

The general functionality and reproducibility of the assay was shown by using different ratios of reduced and oxidized BSA (standard curve). Atlantic salmon (*Salmo salar*) liver tissue and Daubenton's bat (Myotis *daubentoni*) blood samples were used as examples of biological samples.

Standard curve

The standard curve was made by combining various proportions of reduced and oxidized BSA. The carbonyl content of BSA was previously determined with a colorimetric assay. The measurement was conducted with EnSpire and EnVision plate readers. The difference in the slopes is due to the reader settings. Since these settings are constant for the assay runs the calculation of the Carbonyl concentration from the measured signal is unaffected.

[1] The settings of the EnSpire Prototype have been selected to yield comparable signal levels as the EnVision. The production version will have a higher optical efficiency due to the use of filter based excitation for TRF.

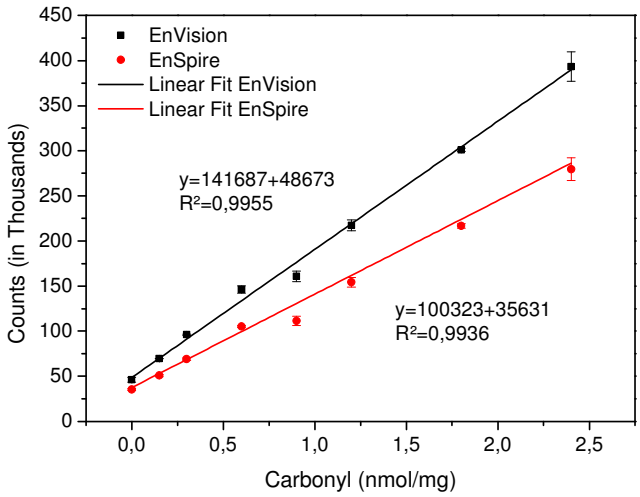

Figure 2. BSA standard curve. The dots represent average count value for triplicate wells and the error bars standard deviation of triplicate wells.

Repeatability

The repeatability of the results of standards was studied by comparing the results from six assays conducted on different days. The repeatability measurements include all steps from the sample preparation to the measurement of the outcome. The coefficient of variation (CV)-values are considered to be excellent as they were derived from assays conducted on different days.

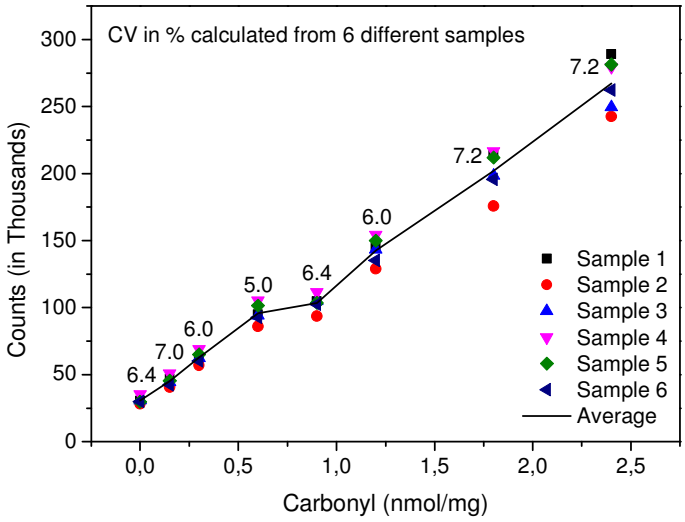

Figure 3. BSA standards from six different assays measured on EnSpire. The CV% of the six standard points is given next to the result. In addition, the graph shows the average result as a continuous line.

Usability verification for biological samples

To show that the assay is working as well with biologically relevant samples, the assay was tested by combining various proportions of reduced and oxidized biological samples (Atlantic salmon liver tissue), treated as BSA (see Materials and Methods, standard preparation). These samples resulted in increased signal proportional to the amount of oxidized sample in some, but not all[2], treated samples.

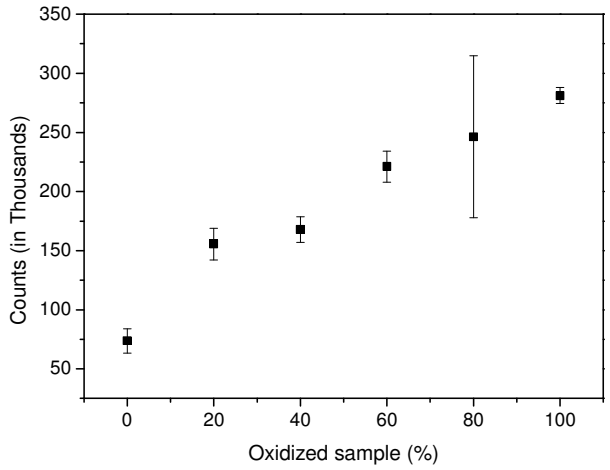

Figure 4. Signal increase in oxidation treated biological sample. The dots represent average count value for triplicate wells and the error bars standard deviation of triplicate wells. (EnVision data shown)

The possible contribution of the nucleic acids to the signal was investigated by comparing the results of same biological samples with or without streptomycin sulfate treatment. The treatment resulted in a small reduction of carbonyl content in three out of five samples tested and therefore the treatment was included in the final assay protocol.

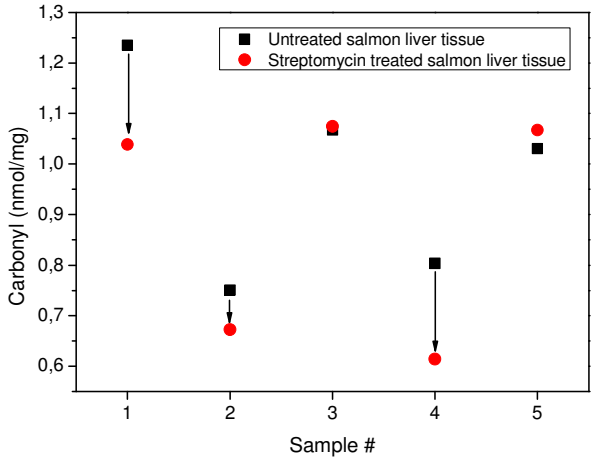

Figure 5. The effect of streptomycin treatment on carbonyl content of five biological samples (EnVision data shown).

[2] We couldn't see the increase in the amount of oxidized sample in all cases, since biological samples are complex and contain lots of different pro- and antioxidants which may counteract or interact with the chemical treatment.

Example results

The method described in this report has currently been applied for biological tissue and blood samples. Figure 6 demonstrates example results obtained from Atlantic salmon liver tissue and Daubenton's bat blood samples. The range of variation is typical for samples from different individuals collected from the wild.

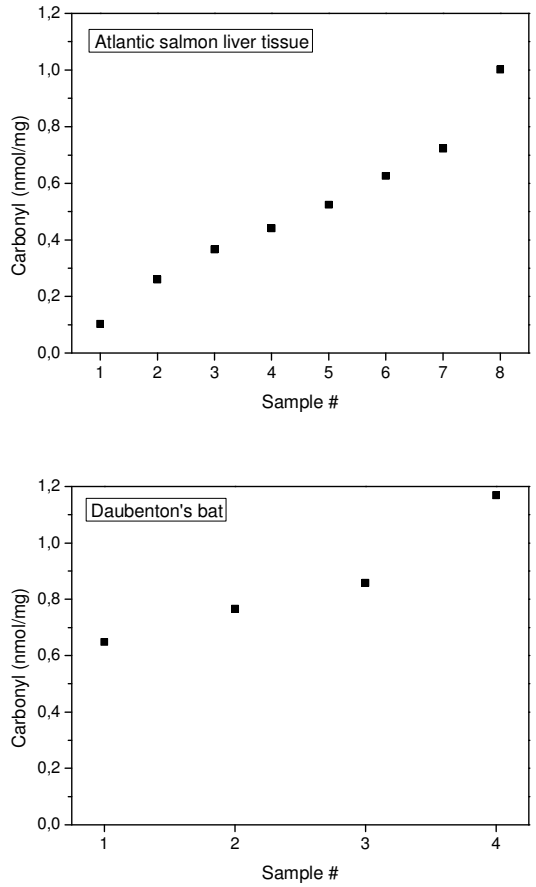

Figure 6. Example variation in carbonyl contents of selected salmon and bat samples determined with the assay. The data shows how different animals and individuals show differences in carbonyl concentration.

Conclusion

The ARP-DELFIA method described in this report is convenient for measuring the large numbers of samples necessary in order to adequately represent biological variation in a study population. The method is also applicable for small sample volumes as only microgram amounts of sample protein were needed for the assay, thus reducing the volume of biological material needed from each specimen. In addition, the use of ARP and europium-labeled streptavidin reduces the steps in the assay and thus the work load per sample when compared to protein carbonyl ELISA method (Buss et al, 1997).

It is possible that some proteins adhere better to the plate and therefore are selected in the assay which can be a potential source of error (Buss et al, 1997). Because carbonylation within proteome is not uniform (Buss et al, 1997; Nystrom, 2005), it is possible that the results may differ from colorimetric or chromatographic

methods' results. However, within the variety of different carbonylation detection methods the plate-based methods may be considered as good general estimates of protein carbonylation levels offering a fast and simple method for screening large sample sets.

A multifaceted approach is often necessary to assess oxidative stress and to understand its consequences for the organism. From the point-of-view of study design, it's important to take into account the nature of the sample and to measure several different indicators of redox status e.g. antioxidant defense and oxidative damage. The described method is currently in use in a study setup employing an array of plate reader assays to study oxidative stress and oxidative damage in ecological samples.

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