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BIOPHYSICS LETTER

FTIR markers of methionine oxidation for early detection of oxidized protein therapeutics

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Abstract The biological activity of therapeutic proteins is strongly dependent on the stability of their folded state, which can easily be compromised by degradation. Oxidation is one of the most common causes of degradation and is typically associated with impairment of the native protein structure. Methionine residues stand out as particularly susceptible to oxidation by reactive oxygen intermediates even under mild conditions. Consequently, methionine oxidation has profound effects on protein activity up to the point of adverse biological responses. Of immediate importance therefore is finding affordable approaches for rapid detection of methionine oxidation before any substantial structural changes can ensue. Herein we report that vibrational bands at 1,044 and 1,113 cm^{-1} in the mid-infrared region can serve as characteristic markers of methionine oxidation in oxidatively stressed protein therapeutics, monoclonal antibodies (IgG1 and its antigen-binding fragment). Such Fourier-transform infrared (FTIR) markers underpin rapid detection assays and hold particular promise for correlation of methionine oxidation with protein structure and function.

Keywords Methionine oxidation · Protein therapeutics (IgG1 and antigen-binding fragment) · Fourier-transform infrared · IR marker bands · Early detection

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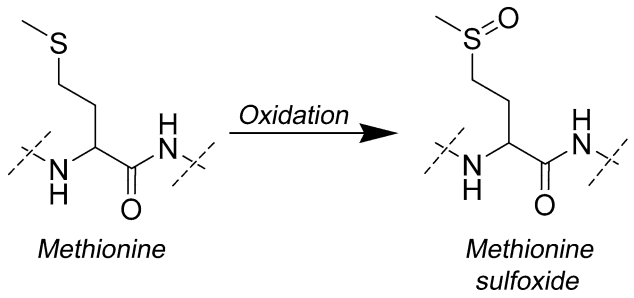
Abbreviations

FTIR	Fourier-transform infrared
LC	Liquid chromatography
MS	Mass spectrometry
CD	Circular dichroism
DSC	Differential scanning calorimetry
NMR	Nuclear magnetic resonance
HDX	Hydrogen–deuterium exchange
ATR	Attenuated total reflectance
ASN	1-Anilinonaphthalene-8-sulfonate
DLS	Dynamic light scattering
mAb	Monoclonal antibody
fAb	Fragment antigen binding
DMSO	Dimethyl sulfoxide

Introduction

Amongst various degradation processes which can adversely affect the activity of protein therapeutics, oxidation can lead to the most profound effects even under mild conditions (Reubsaet et al. 1998). One particular event underpinning the process is the susceptibility of methionine residues to different forms of reactive oxygen (Scheme 1) (Berlett and Stadtman 1997; Carpenter and Manning 2002; Stadtman and Levine 2006; Toennies and Callan 1939). Designed to scavenge environmental oxidants, thereby protecting the activity of a given protein, methionine oxidation can lead to severe effects on protein structure and function (Carpenter and Manning 2002). Establishing approaches that allow detection of methionine oxidation at early stages of protein therapeutics development thus becomes of immediate importance for assessment and monitoring of its impact on protein structure.



Scheme 1 Methionine oxidation reaction

In principle, various techniques may be applied to address this. Liquid chromatography can provide quantitative determination of oxidation levels of peptides and proteins (Berlett and Stadtman 1997; Reubsaet et al. 1998) and, when combined with mass spectrometry (e.g., LC–MS), can aid in identification of oxidation sites following fragmentation by enzymatic digestion (Daugherty and Mrsny 2006; Yan et al. 2007; Zamani et al. 2008). Primarily, these are separation methods and do not give much insight into structural or conformational changes upon oxidation. Although the latter can be monitored by low-resolution molecular biophysics methods such as circular dichroism (CD) spectroscopy (Kelly and Price 2000), fluorescence (Demeule et al. 2007) or differential scanning calorimetry (DSC) (Liu et al. 2006), none of these techniques can reveal specific changes in structure and relate those to specific oxidation sites. Moreover, subtle changes in the microenvironment of aromatic amino acids (tyrosine and tryptophan) may underlie conformational changes, thereby hampering further interpretation (Demeule et al. 2007; Jiang and Narhi 2006; Reubsaet et al. 1998). High-resolution approaches such as nuclear magnetic resonance (NMR) or crystallographic methods, which otherwise can provide the most detailed structural analysis, fail to meet the requirements of rapid on-probe detection and multiple sample screening (Burkitt et al. 2010; Cleland et al. 1993; Houde 2010; Lasch et al. 2001). Mass spectrometry (MS) methods might offer an efficient alternative; for example, hydrogen–deuterium exchange mass spectrometry (HDX/MS) can facilitate assessment of conformational dynamics of intact monoclonal antibodies with resolution down to stretches of just a few residues (Burkitt et al. 2010; Houde 2010). However, the main disadvantage of MS-based analyses remains that they do not provide oxidation information at a glance. Multivariate data analysis is often required to extract structural information from large data sets and to investigate the oxidation rates of methionine at different locations (Zamani et al. 2008). Therefore, MS methods are not deemed appropriate for screening and monitoring of oxidation. All in all, this stimulates the search for a more straightforward and affordable analytical method capable of providing rapid on-probe detection of methionine oxidation. Critical in this regard becomes the ability to obtain qualitative data on oxidation before any detrimental changes in secondary structure can occur. The importance of this cannot be underestimated and becomes particularly crucial at the late and costly stages of therapeutic development (Cleland et al. 1993). Thus, to successfully monitor the process by a single method, it is necessary to have coherent information between methionine oxidation and any related secondary structure alterations. One method which meets this criterion and therefore is advantageous over any of the aforementioned techniques is Fourier-transform infrared (FTIR) spectroscopy. Indeed, FTIR can pinpoint specific chemical modifications and help assess their impact on the global secondary structure of a protein. Combined with its little or no requirement for sample preparation, this makes FTIR an ideal method for assessment of the effect of methionine oxidation on proteins. Surprisingly, however, there have been no reports suggesting use of this technique for the task, nor have there been attempts aiming at identification of a rapid screening platform for methionine oxidation in protein therapeutics. Herein, we address this issue for the application of FTIR for detection of methionine oxidation in monoclonal antibodies.

Experimental procedures

All chemicals were purchased from Sigma–Aldrich unless otherwise stated. mAbs and fAbs in formulation buffer were kindly supplied by GSK (Beckenham, UK) and UCB (Slough, UK), respectively. Protein content was determined using an extinction coefficient of 1.5 ml mg^{-1} cm^{-1} on a high-performance UV–Vis spectrophotometer (Lambda-850; PerkinElmer, MA).

Preparation of oxidatively stressed samples

Samples were buffer-exchanged into a 10 mM sodium phosphate buffer (pH 7.5). Oxidation reactions were then performed at 37°C by addition of hydrogen peroxide to concentration of 150 mM. For FTIR, samples were buffer-exchanged back into 50 mM phosphate buffer (pH 7.5).

FTIR spectroscopy

All FTIR spectra were collected using a Tensor-37 series FTIR spectrophotometer with a BioATR II unit (Bruker Optics, UK) as the sampling platform with a photovoltaic mercury-cadmium telluride (MCT) detector and a Bruker Optics workstation, which was equipped with OPUS

software. Aqueous samples of very low volume (15 µl) were placed in a circular sampling area of radius 2 mm with path length of 6 µm. This multireflection ATR accessory is based on a dual-crystal technology, which has an upper silicon crystal and a hemispherical zinc selenide (ZnSe) lower crystal that does not come into contact with the sample. The temperature of the sample was maintained at 20°C by means of flow connectors to a circulating water bath. This accessory was purged continuously with dry nitrogen throughout the experiment via telescopic inserts that seal the optical path inside the spectrometer sample compartment. All FTIR spectra were collected with resolution of 4 cm^{-1}, scanner velocity of 20 kHz, 256 scans, phase resolution of 32, and zero filling factor of 4.

CD spectroscopy

All CD spectra were obtained on a Jasco J-810 spectrophotometer using the following parameters: standard sensitivity, data pitch of 0.1 nm, scanning speed of 50 nm/min, bandwidth of 1 nm, response time of 1 s, and six accumulations for far UV and four accumulations for near UV. Samples at 0.5–1 mg/ml protein concentrations were used.

Fluorescence spectroscopy

Tryptophan fluorescence was measured with a LS 55 fluorimeter (PerkinElmer Instruments, MA) at 25°C in a 1-cm quartz cuvette (Hellma GmbH, Germany) and 0.06 mg/ml protein solution. Spectra were recorded, normalized with background fluorescence subtracted. Samples were excited at 295 nm, and the emission spectra were measured between 280 and 420 nm. The excitation and emission slits for the nonstressed sample were 5 nm, and for the stressed sample, the excitation and emission slits were changed to 2.5 nm; the slits were reduced to avoid saturating the detector. ANS binding assays were performed under the same conditions, except samples were excited at 380 nm and the emission spectra were measured between 400 and 600 nm. The excitation and emission slits were 5 and 10 nm, respectively.

Dynamic light scattering

DLS batch measurements were carried out on a Zetasizer Nano (ZEN3600; Malvern Instruments, Worcestershire, UK) in a low-volume disposable cuvette at 25°C. No filtration of the sample was carried out before the measurements, and therefore the aggregate population was not affected. The data were analyzed using the manufacturer's Dispersion Technology Software (DTS version 5.10).

Peptide synthesis

The model peptide (ac-AAMAA-am) was assembled on a Liberty microwave synthesizer (CEM Corporation) using standard Fmoc solid-phase protocols. The peptide was purified by reversed-phase high-performance liquid chromatography (HPLC). The masses of nonoxidized and oxidized forms were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry (MS): ac-AAMAA-am, *m/z* 474.2 (calcd., $[M + H]^+$), 497.1 (found, $[M + Na]^+$); ac-AAM(O)AA-am, *m/z* 490.2 (calcd., $[M + H]^+$), 513.4 (found, $[M + Na]^+$).

Results

FTIR principally relies on the vibrations of chemical bonds at characteristic frequencies, thus allowing analysis of chemical bonding failures. Given this dependence, a monoclonal antibody (mAb) was subjected to oxidation under accelerated stability conditions to induce shifts in absorption bands. Indeed, appreciable bands detected at 1,044–1,113 cm^{-1} were observed for oxidized samples (Fig. 1a). The obtained profile was consistent with that of the FTIR spectrum for dimethyl sulfoxide (DMSO) (Fig. 2). DMSO shows a sharp peak at 1,044 cm^{-1}, implying that the band relates to oxidized methionine, i.e., methionine sulfoxide. The band derives from S=O stretching vibration, and the results obtained are in good agreement with observations made in other proteins (Lasch et al. 2001). Furthermore, bands at 1,044 cm^{-1} and 1,113 cm^{-1} were also recorded for the oxidized antigen-binding fragment (fAb) of the immunoglobulin, suggesting no impact of carbohydrate moieties on the spectra of mAb (Fig. 3), normally associated with strong bands in the 900–1,300 cm^{-1} region for crystallizable fragment (Fc) (Khajehpour et al. 2006). Additionally, to confirm the effect of oxidation at the primary structure level without the contribution of conformational changes, a model peptide, ac-AAMAA-am, was probed. The peptide has a single methionine residue forming two peptide bonds with flanking alanines, thus mimicking native primary structure environments. Gratifyingly, the spectra recorded for oxidized ac-AAMAA-am showed the emergence of the same characteristic band around 1,044 cm^{-1}, which in conjunction with the observed mass increase of 16 Da when compared with the nonoxidized form, indicates the direct correlation between the band and the oxidation of the methionine residue (Fig. 4).

To assess the effect of oxidation on the global secondary structure of the protein, the second-derivative spectra of nonstressed and oxidatively stressed mAb were taken,

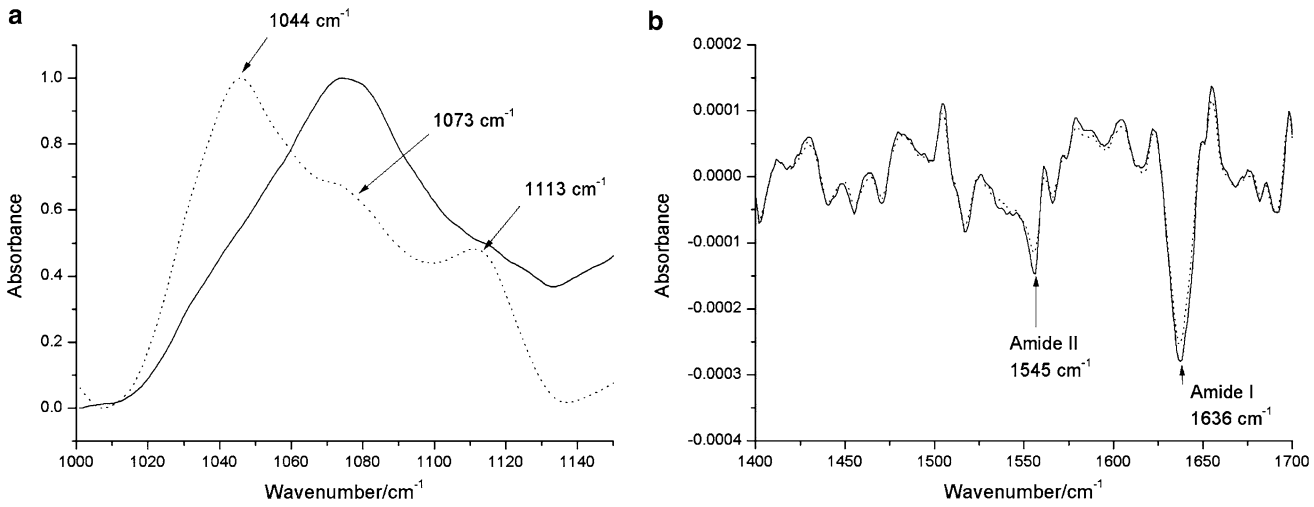


Fig. 1 FTIR analysis of oxidatively stressed mAb. **a** Spectra for mAb (10 mg/ml) (nonstressed, *solid line*) and after 1 h of incubation with H_2O_2 (150 mM at 37°C) (stressed, *dotted line*) in phosphate buffer (50 mM, pH 7.5). **b**. Second-derivative spectra of nonoxidized (*solid line*) and oxidized (*dotted line*) mAb showing amide I and II bands. All spectra are min–max normalized

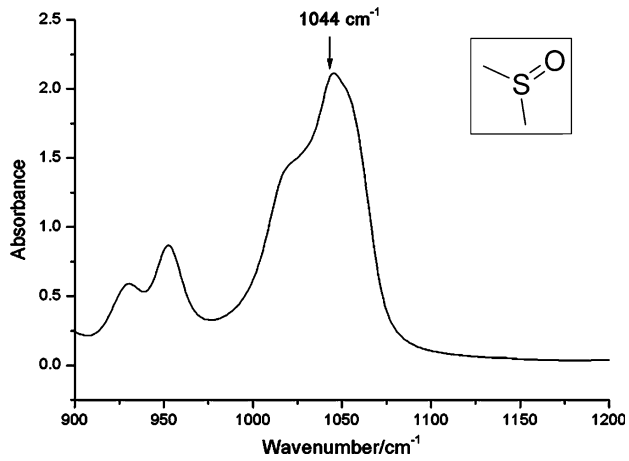


Fig. 2 FTIR spectrum of dimethyl sulfoxide

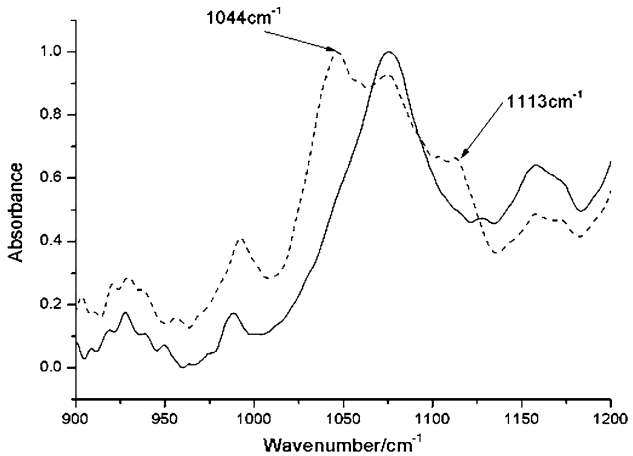


Fig. 3 FTIR spectra (min–max normalized) of fAb in phosphate buffer (native, *solid line*) and after 1 h of oxidation (150 mM H_2O_2 at 37°C) in phosphate buffer (oxidized, *dashed line*)

revealing no change in the amide I and II bands. In both cases, amide I band at 1,636 cm^{-1} was indicative of a β-sheet structure (Haris 1999), which is the native conformation of the mAb (Liu et al. 2008). Complementary to this were far-UV (fUV) CD spectra of nonoxidized and oxidized fAb with a minimum at 217 nm and a maximum around 200 nm, also supporting the native β-sheet structuring of the protein (Fig. 5a) (Vermeer and Norde 2000). However, subtle differences in fUV CD spectra around 228 nm were observed for the oxidized fAb (Fig. 5a). At this wavelength, the second tryptophan absorption envelope is known to peak (Demeule et al. 2007). Consistent with this, near-UV (nUV) CD spectra for nonoxidized and oxidized fAb samples showed maxima at 290–285 nm attributable to tryptophan, and featured signatures from 260 to 280 nm due to tyrosine, phenylalanine, and disulfides (Fig. 5b) (Jiang and Narhi 2006). The results indicate that the environment of the chromophores, aromatic amino acids and disulfide bonds, of the samples are essentially the same, which in turn confirms the overall tertiary structure as unchanged. Further support for this came from the fluorescence spectra of the samples. Two maxima at 332 and 348 nm indicative of tryptophan residues existing in two different environments, with the maximum at 332 nm corresponding to a more hydrophobic environment (Creighton 1997), were apparent for both oxidized and nonoxidized fAb (Fig. 6). No shift was recorded in the maxima of the oxidized fAb. This implies that there was no change in the tryptophan environment and therefore no change in the overall tertiary structure of the protein. However, methionine oxidation may cause local changes in structure, albeit small, but sufficient to lead to a hydrophobic imbalance of the mAb. With this in mind, we performed a binding assay with

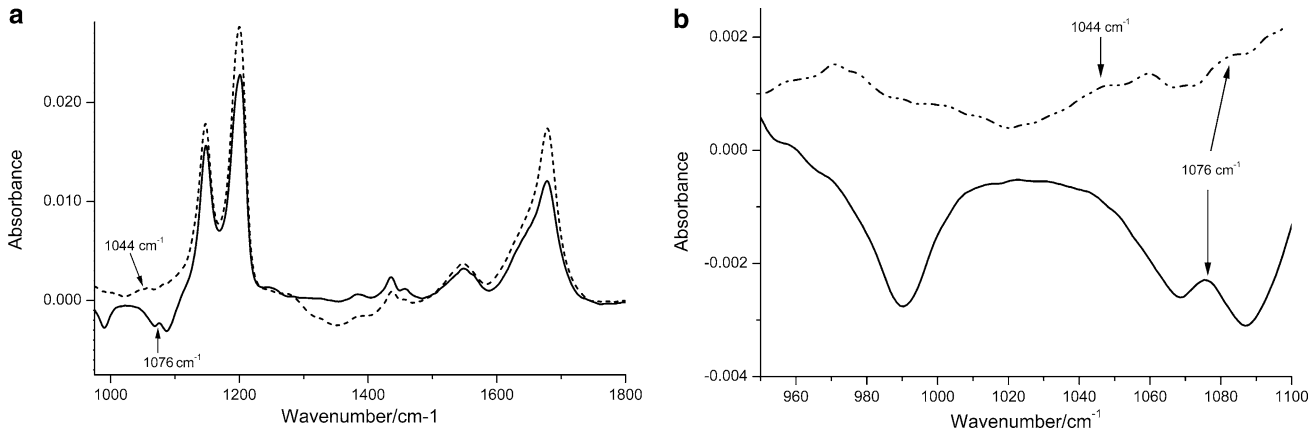


Fig. 4 FTIR spectra (min–max normalized) of model peptide ac-AAMAA-am in phosphate buffer (native, *solid line*) and after 1 h of oxidation (150 mM H_2O_2 at 37°C) in phosphate buffer (oxidized, *dashed line*)

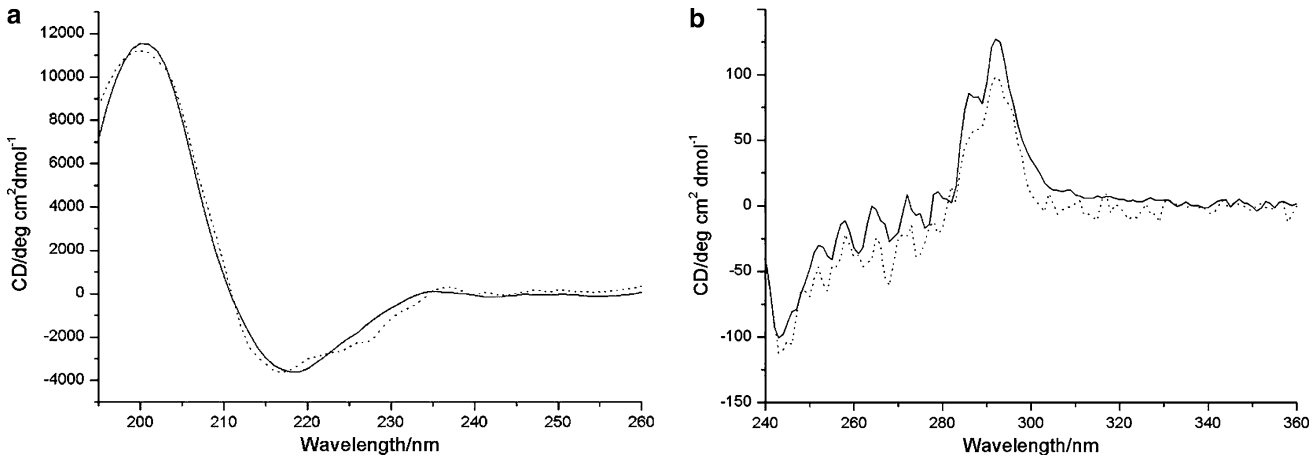


Fig. 5 **a** fUV spectra of the fAb (nonstressed, *solid line*) and the oxidized fAb (*dash line*) in phosphate buffer. fUV CD spectrum was obtained in a 0.01-cm cell with data pitch of 0.5 nm, scan speed of 100 nm/min, and accumulation of six scans. **b** nUV spectra of the fAb (8.9 mg/mL, nonstressed, *solid line*) and the oxidized fAb (2.4 mg/mL, stressed, *dot line*) in phosphate buffer. nUV CD spectrum was obtained in a 0.1-cm cell with data pitch of 0.5 nm, scan speed of 100 nm/min, and accumulations of six scans

1-anilinonaphthalene-8-sulfonate (ANS). ANS is used as a fluorescent conformational probe that intensely fluoresces in hydrophobic environments but not in hydrophilic regions. The fAb molecules have hydrophilic surfaces, and therefore ANS interaction is expected to be very little or none. In marked contrast, an increase in the fluorescence intensity of the stressed fAb should be observed if the oxidation led to enhanced exposure of hydrophobic regions of the protein. Indeed, this was supported by the assay, which revealed notably stronger binding of ANS to the surfaces of the oxidized fAb (Fig. 7). Such an enhancement in hydrophobicity may lead to increased aggregation propensity. Dynamic light scattering experiments, however, showed only a single, monodisperse component with hydrodynamic radius of ~7 nm in both samples, confirming the local character of structural changes with no tendency for aggregation (Fig. 8) (Jiang and Narhi 2006; Nobbmann et al. 2007).

Discussion

The observation of the bands in the FTIR spectra of the stressed mAb and fAb is consistent with methionine oxidation. The DMSO spectrum confirmed that the 1,044 cm^{-1} band is due to the stretching vibration of the sulfoxide group. Admittedly, the origin of the 1,113 cm^{-1} band is less clear. Related to oxygen-evolving complexes, the band can be attributed to an oxidation event, the exact nature of which requires separate and more detailed study (Mizusawa

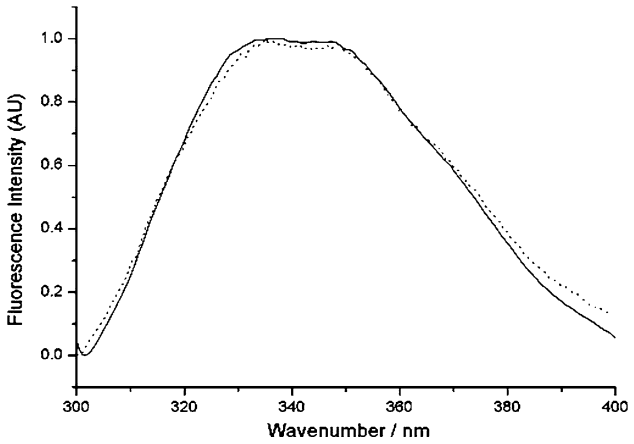


Fig. 6 Intrinsic fluorescence spectra of the fAb before (nonstressed, *solid line*) and after (stressed, *dot line*) incubation with H_2O_2 (150 mM, 1 h at 37°C) in phosphate buffer (50 mM, pH 7.5)

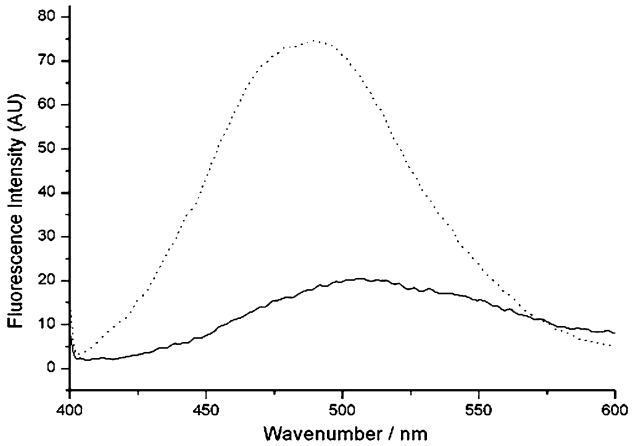


Fig. 7 ANS binding assay. Fluorescence spectra of the fAb with tenfold molar excess of ANS before (nonstressed, *solid line*) and after 1 h of incubation with H_2O_2 (150 mM at 37°C, stressed, *dot line*) in phosphate buffer (50 mM, pH 7.5)

et al. 2004). Nevertheless, the oxidation of the same mAb molecule has been studied using HDX/MS, confirming the exclusive oxidation of methionine residues (Burkitt et al. 2010). In this regard, it is reasonable to assume the coherent appearance of the two bands as FTIR markers of methionine oxidation. With the same bands observed for both mAb and fAb after incubation with H_2O_2, subtle structural changes in the microenvironment of the fAb were recorded by fUV CD. The observation was further supported by intrinsic and ANS fluorescence to support the view of oxidation leading to hydrophobic imbalance caused by local conformational changes in tryptophan microenvironments. DLS measurements confirmed the monodispersity of the oxidized samples, suggesting no immediate aggregation of the protein.

Collectively, the obtained results indicate that the detection of the bands before the occurrence of substantial structural changes provides early detection markers of

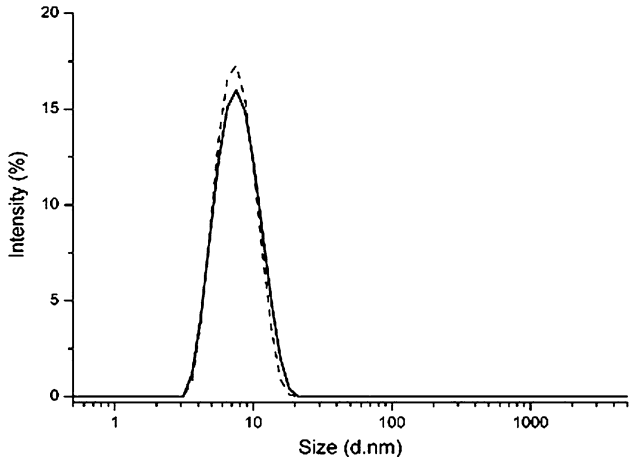


Fig. 8 Dynamic light scattering of the fAb (1 mg/mL) before (nonstressed, *solid line*) and after 1 h of incubation with H_2O_2 (150 mM at 37°C, stressed, *dot line*) in phosphate buffer (50 mM, pH 7.5)

protein oxidation and subsequently of other associated degradation processes such as aggregation. This constitutes a significant advantage over other methods currently used to detect oxidation of protein therapeutics, which cannot provide consistent information on the oxidation and the overall conformation of the protein. In contrast, FTIR spectroscopy offers a straightforward platform for rapid detection assays, holding promise for correlation of the oxidation effect with protein structure and function.

Conclusions

The obtained results highlight the application of FTIR as a screening technique for oxidation of protein therapeutics. The bands at 1,044 and 1,113 cm^{-1} were revealed to be sensitive markers for detecting an oxidation event in a monoclonal antibody prior to any substantial alterations in folding. The local environmental change due to the oxidation of methionine has been confirmed by CD and fluorescence spectroscopy for the antigen-binding fragment. However, technical constraints associated with these biophysical techniques limit their use in therapeutic analysis. FTIR, which overcomes these barriers, can therefore be exploited earlier in the development cycle and hence facilitate better design and selection of protein drugs. The main advantage of FTIR-based assays is that they can give insight into folding characteristics and oxidation simultaneously, unlike other techniques, and could easily be exploited in any stage of the therapeutic development.

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