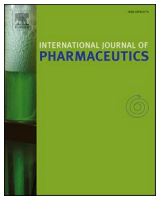
International Journal of Pharmaceutics 654 (2024) 123926

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm



Towards a better understanding of light-glucose induced modifications on the structure and biological activity of formulated Nivolumab

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ARTICLE INFO

Keywords:
monoclonal antibody (mAb)
Glucose Degradation Products (GDPs)
5-hydroxymethyl-2-furfural (5-HMF)
Protein photostability
Chemical oxidation
Protein aggregation

ABSTRACT

In the last years, monoclonal antibodies (mAbs) have rapidly escalated as biopharmaceuticals into cancer treatments, mainly for their target specificity accompanied by less side effects than the traditional chemotherapy, and stimulation of reliable long-term anti-tumoral responses. They are potentially unstable macromolecules under shaking, temperature fluctuations, humidity, and indoor and outdoor light exposure, all stressors occurring throughout their production, transport, storage, handling, and administration steps. The chemical and physical modifications of mAbs can lead not only to the loss of their bioactivity, but also to the enhancement of their immunogenicity with increasing risk of severe hypersensitivity reactions in treated patients because of aggregation. The photostability of Nivolumab, the active principle of Opdivo®, has been here studied. The chemical modifications detected by LC-MS/MS after the light stressor showed Trp and Met mono and double oxidations as primary damage induced by light on this mAb. The oxidations were stronger when the mAb was diluted in sterile glucose solution where 5-HMF, a major heat glucose degradation product, acted as singlet oxygen producer under irradiation. However, no significant changes in the mAb conformation were found. On the contrary, formation of a significant extent of aggregates has been detected after shining high simulated sunlight doses. This again took place particularly when Nivolumab was diluted in sterile glucose, thus raising a direct correlation between the aggregation and the oxidative processes. Finally, the biological activity under light stress assessed by a blockade assay test demonstrated the maintenance of the PD-1 target recognition even under high light doses and in glucose solution, in line with the preservation of the secondary and tertiary structures of the mAb. Based on our results, as sterile glucose is mostly used for children's therapies, special warnings, and precautions for healthcare professionals should be included for their use to the pediatric population.

1. Introduction

Monoclonal antibodies (mAbs) have revolutionized modern therapeutic treatments and in these last years are one of the most rapidly growing drug classes. These drugs have particularly escalated as biopharmaceuticals into cancer treatments, mainly for their target specificity and stimulation of reliable antitumoral responses (Zahavi and Weiner, 2020). Indeed, compared to small molecule drugs, mAbs possess several advantages, such as increased specific recognition of conformational structures of proteins with more expected strict selectivity and affinity for therapeutic purposes, decreased off-target effects and prolonged half-life in the bloodstream, because renal excretion is limited to low molecular weight compounds (Tsumoto et al., 2019). Since the eighties, over 100 mAbs have been authorized as drugs by the FDA, able to treat a variety of complex conditions in a more targeted and personalized approach. "Immune checkpoint blockade" is the therapeutic strategy based on mAbs by enhancing T cell response (Hargadon et al., 2018). Optimized handling and storage are critical to ensure the quality, safety, and efficacy of mAb products, as well as to guarantee the wide scale use of mAb therapeutics in patients ("Guideline on similar biological medicinal products containing monoclonal antibodies – non-clinical and clinical issues," n.d.).

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https://doi.org/10.1016/j.ijpharm.2024.123926
Received 16 November 2023; Received in revised form 30 January 2024; Accepted 18 February 2024
Available online 22 February 2024

During their real-life, they are potentially unstable under the multiple stressors that can occur throughout all stages of their production, transport, storage, handling, and administration, i.e., shaking, temperature fluctuations, humidity, and light exposure (Nejadnik et al., 2018). The consequent physical and chemical modification of mAbs can lead not only to the loss of their bioactivity (Haberger et al., 2014), but also to the enhancement of their immunogenicity, with increasing risks of severe hypersensitivity reactions (Nabhan et al., 2020). Additionally, mAbs administered intravenously are diluted in 0.9 % NaCl or in 5 % glucose solutions and consequently, the diluted protective excipients decrease their specific role of defense, i.e., against oxidative amino acid modifications or aggregation (Le Basle et al., 2020). Therefore, chemical modifications, including asparagine (Asn) deamidation, aspartic acid (Asp) isomerization and methionine/tryptophan (Met/Trp) oxidation are easily expected to occur as in common proteins specifically when exposed to light during handling at hospitals and pharmacies, i.e., dispensing the drug product from one container (e.g., vial) to another (e.g, syringe, IV-bag), dilution and reconstitution of lyophilized drug product. Therapeutic proteins may be exposed to both visible and UV light and may undergo a change in structure, chemistry, and functional properties that could have over time a potential impact on safety and ultimately lead to a drop in their biological potency (Manning et al., 2010; Sreedhara et al., 2016). The main modifications under light are oxidation, crosslinking, and fragmentation. Especially aromatic residues are very sensitive to light, although light exposure does not seem to directly alter mAbs secondary and tertiary structures. The main mechanisms under light exposure include direct UV light absorption generating singlet or triplet excited states of proteins or radicals' formation followed by oxidation or photosensitization by internal or external chromophores present in the formulation. Free radicals can form by electron transfer or hydrogen abstraction generating peroxyl radicals able to further attack the amino acid side chains. Tyrosine, histidine, and tryptophan peroxides propagate protein oxidation and trigger further oxidation via both radical and non-radical mechanisms. Moreover, decomposition of hydroperoxides to radicals can ultimately lead to backbone fragmentation (Davies, 2005). Among the most photo-oxidation vulnerable residue there is Tryptophan. Under the effect of light, it undergoes modification to stable photoproducts such as kynurenine and N-formyl-kynurenine which have shown photosensitizing properties, higher than tryptophan, and further amplify the damage to the entire protein structure (Ulbricht et al., 1984). However, even another aromatic amino acid, histidine, has shown to be prone to photooxidation and photoaddition to mAbs and His-His crosslinking resulting from the nucleophilic attack of a His to a photooxidized His from another protein but also photoadducts have been detected(Lei et al., 2021).

In general, it has been seen a correlation between the loss of stability (DSC), generation of aggregates (HMWS), and loss of function (Shah et al., 2018). To warrant the standards of quality, safety, and efficacy, the Q10 document ("Pharmaceutical Quality System Q10," 2008) of the International Conference for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), has drawn several stability tests that must be ran on drug materials and products before entering the market. The guidelines contained in ICH Q1B indicate how to carry out photostability tests on therapeutic proteins and to protect them from photooxidation by adding photosensitizers to the drug formulation such as polysorbate 80 (Agarkhed et al., 2013). Specifically, the ICH Q5C is the document that regulates and defines the parameters to evaluate the quality of biotechnological products. Therefore, a careful analysis of the stability-influencing factors on mAbs may help to avoid or mitigate instability problems, such as unfolding, aggregation, with chemical modifications that can lead to a loss of protein conformation during the real life of a pharmaceutical protein. Such undesired degradation reactions could also alter the ability of mAbs to recognize their target and consequently the therapeutic approach on patients could be unsuccessful.

Here we have systematically irradiated in a sunlight simulator a therapeutic mAb, Nivolumab, in its formulation Opdivo® and in two diluting conditions, 0.9 % NaCl and 5 % glucose, used before patient administration. Formulated Nivolumab is widely used in therapy for the treatment of various types of cancer, both in mono and in poly-therapy, with excellent responses from treated patients. It is mainly used for the treatment of patients with advanced (unresectable or metastatic) melanoma in adults, alone or in combination with Ipilimumab. It is also designated for the treatment of locally advanced or metastatic non-small cell lung cancer (NSCLC) after prior chemotherapy in adults or in monotherapy for the treatment of advanced renal cell carcinoma after previous therapy in adults; finally, it is used for the treatment of adult patients affected by relapsed or refractory classical Hodgkin lymphoma (cHL) after autologous stem cell transplantation (ASCT). Structurally, Nivolumab is a humanized IgG4 antibody, formed by 1308 residues, with a molecular weight of ~ 144 kDa and with an isoelectric point of 7.92 (Fig. 1) (Tan et al., 2017). The complex with its target PD-1 has been studied by crystallography (Zak et al., 2017) and recently by high resolution mass spectrometry (Zhang et al., 2020). Nivolumab binds PD-1 by its heavy chain (VH) and light chain (VL), and their interaction involves all three CDR loops in Nivolumab VH region, and less the CDR2 and CDR1 loops in VL.

We investigated the chemical and physical modifications induced on Nivolumab in its reconstituted form, or in diluted ready to administer solutions, by different doses of artificial sunlight. Particularly interesting was to study the effect of glucose degradation products (GDPs), i.e., 5-hydroxymethyl-furfural (5-HMF), present in the sterile glucose solution with respect to the 5 % non-sterile glucose, and to the 0.9 % saline solution used for the dilution of the formulated mAb. It has been already demonstrated that 5-HMF, besides its acute toxicity under chronic administration (Li et al., 2020), is able to induce the production of singlet oxygen under irradiation, dramatically affecting the photostability of the drug, as demonstrated for catecholamines (Brustugun et al., 2005). The photo destabilizing effect of 5-HMF on Nivolumab was measured in the concentration range from 3.16 μg/ml, corresponding to the concentration of 5-HMF found in the commercially glucose infusion solution used, to 22.6 μg/ml of 5-HMF, that is the highest concentration allowed by the British Pharmacopoeia in glucose solutions sterilized by heat. The influence of sterile glucose solution or dilution in saline solution in combination with light on Nivolumab biological activity was also investigated to predict the efficacy of the mAb in different stress conditions (i.e., light and dilution).

Although the exposure of formulated mAbs to high doses of external or internal light seems not common during transport or preparation of the IV bag dilution, this is of outmost importance during the long administration (hours) to the patient or in case of home delivery and storage. It is a matter of fact that light strongly impacts on mAbs stability as previously reported (Fongaro et al., 2022).

2. Material and methods

Material. Nivolumab (Bristol-Myers Squibb, 10 mg/mL,) was kindly provided by Istituto Oncologico Veneto (IOV). Sterile Glucose 5 % (Eurospital, Italy) was purchased in a public pharmacy. 5-Hydroxymethyl-2-furaldehyde (5-HMF) was purchased from Sigma-Aldrich, USA. All other reagents of analytical grade were obtained from Merck (Darmstadt, Germany).

Stress experiments: light and dilution. Nivolumab was tested after exposure to different artificial solar radiation doses to simulate routine handling from the site of manufacture to patient administration and home delivery and storage. The doses of light shined to the samples simulated different times of sunlight exposure, i.e., 1.5 days (confirmatory test, ICH guidelines), 1 week- and 3 weeks (forced test). Furthermore, the perturbing effect of the dilutions chosen has been considered, reproducing a real situation of drug administration. The conditions taken into consideration in these experiments were:

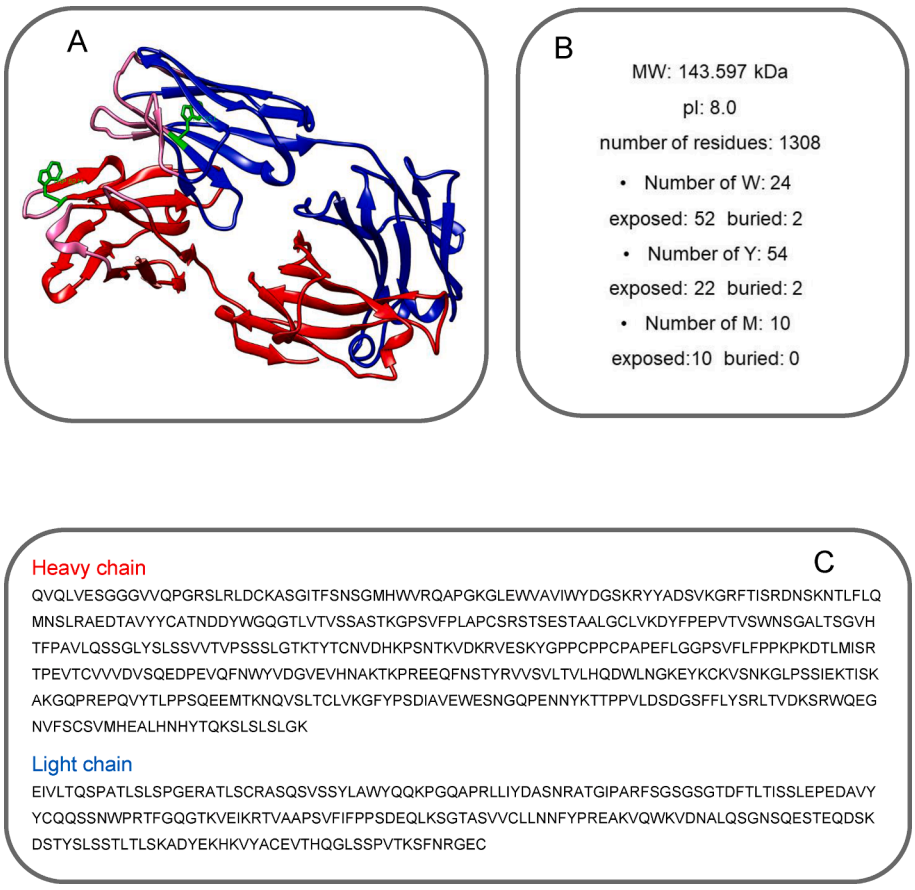

Fig. 1. Three-dimensional structure of Nivolumab (PDB code 5GGQ) (A). The structure was obtained by using the Chimera software. CDRs are indicated in pink. Trp residues susceptible to oxidation and located close or belonging to CDRs are highlighted. Amino acid sequence of Nivolumab (B). Structural information of Nivolumab (C). CDRs regions were identified by using AbRSA web service (Li et al., 2019). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nivolumab/Opdivo® formulation as such (10 mg/mL); Nivolumab/Opdivo® diluted with sterile 5 % glucose solution (2 mg/mL); Nivolumab/Opdivo® diluted with non-sterile 5 % glucose solution (2 mg/mL); Nivolumab/Opdivo® diluted with 0.9 % NaCl saline (2 mg/mL). After the light stressor, all the samples were further diluted to a final concentration of 0.1–0.2 mg/mL to be examined by the different analytical techniques.

Sun test. Photostability tests were performed on a SunTest CPS+ (Atlas Technologies GmbH, Linsengericht, Germany). The instrument was equipped with a 1.8 kW xenon lamp. The doses of UV energy used were 720 KJ/m^2 (200 W hours/m^2) and 3360 KJ/m^2 (200 W hours/m^2), with an irradiance of 360 mW/cm^2 (300–800 nm). The first dose corresponded to the amount of light that the sample would take approximately in a day's exposure for confirmatory testing. The other one is used for forced degradation testing. Non-irradiated samples (dark), that have received the same treatment as the irradiated ones (same batch, same dilutions, same diluents), covered with aluminium foil in dark vial, were used as controls. The temperature in the SunTest was between 20 and 25 °C. All the samples laid on a water refrigerated plate to reduce the temperature due to irradiation. The analyses were performed in accordance with the ICH Q1B guidelines ("Stability testing: photostability testing of new drug substances and products Q1B").

2.1. Spectroscopic studies

UV–Vis absorbance analysis was carried out with a Cary 50 spectrophotometer (Agilent, Santa Clara, CA), acquiring spectra from 230 to 350 nm, at a scan rate of 300 nm/min. Quartz cuvettes of 3 mL and with 1 cm pathlength were used. The buffer contribution was subtracted. Second derivative was obtained by using the software provided by the manufacturer.

Singlet oxygen was determined according to Kraljić (Kraljić and Mohsni, 1978). Samples were added to p-nitrosodimethylaniline (RNO) (4 x 10^{-5} M) and imidazole (4 x 10^{-5} M) in 0.02 M phosphate buffer, pH 7.3, and irradiated with increasing artificial sunlight, or UVB or UVA light doses. The absorbance at 440 nm was measured after each treatment. Data were expressed as the percentage of RNO bleaching.

Fluorescence measurements were performed with Varian Cary Eclipse spectrofluorometer (Agilent, Santa Clara, CA). Samples were excited at 280 nm and emission spectra were recorded from 300 nm to 500 nm at a scan speed of 120 nm/min, with quartz cuvettes with 1 cm pathlength. Excitation and emission slits were set to 5 nm. The spectra were obtained at protein concentration of 0.1 mg/mL, diluting Nivolumab in 0.9 % NaCl, in 5 % sterile and non-sterile glucose to 1 mg/ml, just before measurements. The contribution of 5-HMF at 280 nm was corrected and its concentration was calculated in commercial sterile glucose as 3.16 μg/mL. Baseline contribution was removed by its subtraction from protein sample spectra. Measurements were conducted in triplicates. The instrument calibration is routinely performed according to the procedure provided by the manufacturer (Agilent, Santa Clara, CA).

Circular dichroism (CD) measurements were performed on a JASCO spectropolarimeter (model J-810, Tokyo, Japan). Spectra were acquired in the near (350–250 nm) and in the far (198–250 nm) UV range, at a scan speed of 20 nm/min, collecting data with high-tension voltage < 600 V and avoiding too noisy signals. A quartz cuvette (0.5 or 0.1 cm,

respectively) was used. All protein samples were measured at the same settings averaged in five and the buffer data subtracted. The concentration of samples was 0.5 or 0.1 mg/mL. The mean residue ellipticity [θ] (degree cm^2dmol^{-1}) was calculated from the formula [θ] (θ_{obs}/10) (MRW/lc), where θ_{obs} is the observed ellipticity in degrees; MRW is the mean residue molecular weight of the protein; l is the optical path length in cm; and c is the protein concentration in g/mL.

Dynamic Light Scattering (DLS) analysis was conducted by a Zetasizer Ultra (ZSU5700, Malvern instruments, Worcestershire, UK). Sample aliquots were loaded at 1 mg/mL. Each measurement was performed in triplicate. Data were analysed with ZS Xplorer software and expressed as intensity. The appropriate attenuator position was automatically determined by the Zetasizer instrument during the measurement sequence. The sensitivity of this instrument was 0.01 nm.

2.2. Aggregation analysis

Size exclusion chromatography (SEC) was carried out on a High-Performance Liquid Chromatography (HPLC) (Jasco HPLC LC-1500, Tokyo, Japan) with a TSKgel® UP-SW3000 column (4.6 × 300 mm, 2 μm; Tosoh Bioscience, Japan). The elution was conducted by 0.1 M sodium phosphate pH 6.9, containing 0.1 M Na_2SO_4, at a flow rate of 0.350 mL/min, detecting at 280 nm. Sample aliquots were loaded at a concentration of 1 mg/mL. The calibration of the column was obtained by loading a mixture of proteins with known molecular weight. Quantification was done by OriginPro 2018b software, integrating the area under the peaks after the baseline subtraction. The areas (Au) of the monomer and high molecular weight (HMW) species were calculated from SEC profiles and expressed as percentage of aggregated species (AuHMW/AuTOT × 100).

SDS PAGE. Ready prep gels (4–12 %) were purchased from Invitrogen (Thermofisher Scientific Van Alley, Carlsbad, CA). 3 μg of antibody was loaded per well in 50 mM Tris-HCl, pH 6.8, containing 2 % SDS, 2 % beta-mercaptoethanol, and 10 % glycerol. The electrophoretic run was conducted at 80 V for 5 min and then at 120 V for about 1 h. Staining was done by Coomassie Blue R 250 in methanol, acetic acid, and deionized water (5:4:1 ratio). Destaining was carried out with solution of ethanol, acetic acid, and water (1:1.5:7.5). Images were acquired with a Perkin Elmer Geliance 600 Imaging Systems.

LC-MS/MS analysis. Fingerprinting analysis was carried out by a Xevo G2-XS ESI-Q-TOF mass spectrometer (Waters Corporation, Milford, Massachusetts, US). Lyophilized protein samples were solubilised in 6 M guanidinium-HCl, 50 mM Tris-HCl pH 8.5 and incubated for 1 h 30 min at 40 °C with Tris (2-Carboxyethyl) phosphine (TCEP) at 1:10 ratio with the cysteine residues. The alkylation of Cys residues was obtained with Iodoacetamide, added in a 1:10 ratio with the cysteine residues and incubated for 30 min in the dark. The buffer was changed with 100 mM ammonium bicarbonate by Amicon® Ultra 0.5 mL centrifugal filters (Merck Millipore Ltd., Ireland). Tryptic digestion was conducted at a 1:20 trypsin to mAb ratio (w/w). The reaction was left at 37 °C overnight and then stopped by freezing at −20 °C. Proteolytic mixtures were analyzed by LC-MS/MS and the parameters were set according to samples (1.5–1.8 kV capillary voltage and 30–40 V cone voltage). The elution was performed at a flow of 0.2 mL/min eluted with the following acetonitrile/0.1 % formic acid - water/0.1 % formic acid gradient: 2–65 %, 36 min, 65–98 %, 2 min. Data were processed using BiopharmaLynx software, setting cysteine carbamidomethylation as a fixed modification and W- and M−oxidation (mono and double) as variable modifications.

Biological assay. The biological activity was evaluated by a PD-1/PD-L1 Blockade Bioassay kit (Promega, Fitchburg, WI, USA). PD-L1 aAPC/CHO-K1 cells were seeded in 96-well plates at a density of 5 × 104 /well in the complete culture medium. After few hours, the medium was removed, and 1 × 105 PD-1 Jurkat cells were added to each well with test inhibitors. The cells were cultured overnight at 37 °C. Serial dilutions of stressed mAbs samples were prepared starting from a 25 μg/mL. 40 μL of suspension was transferred into the 60 wells and incubated at 37 °C, 5 % CO_2 for 6 h. Finally, luciferin substrate (Bio-Glo reagent) was added and incubated for 5 min and luminescence was quantified using Victor3 PerkinElmer.

3. Results

3.1. Diluent sensitivity to light and production of singlet oxygen

This study is focused on the photostability of formulated Nivolumab, therefore the influence of the diluents, in particular glucose, has been evaluated in detail. Commercially available glucose solutions generally contain 5-HMF (3–5 μg/ml), generated as degradation product, during the heat sterilization process (Kowalski et al., 2013). As regards sterile glucose use in intravenous therapeutics, the British Pharmacopoeia reports a limit of concentration up to 22.6 μg/ml of 5-HMF to avoid general toxicity (Bryland et al., 2010), being very reactive towards amino groups and amino acids (Nikolov and Yaylayan, 2011). However, Zhao et al. (Zhao et al., 2013) have demonstrated antioxidant and antiproliferative properties of 5-HMF in vitro. On the contrary, under light, 5-HMF can produce significant amounts of singlet oxygen able to react with the drugs solubilized in sterile glucose (Brustugun et al., 2005).

The sterile (SG) and non-sterile glucose (NSG) solutions were characterized by UV–Vis absorbance spectroscopy (Fig. 2) to evaluate their contribution in the spectroscopic properties of Nivolumab when dissolved in these diluents both before and after light exposure. Due to the presence of heat glucose degradation products, mainly 5-HMF, sterile glucose showed a main absorption band centered at 284 nm (Fig. 2A-C, black line), which was not present in the non-sterile glucose (Fig. 2D). To monitor the photostability of these diluents, they were irradiated under increasing doses of simulated solar light, UVA or UVB light. UVA light did not affect the absorbance spectrum of sterile glucose (Fig. 2B, purple line), while the solution appeared highly unstable under UVB light (Fig. 2C) and to a lesser extent under sunlight (Fig. 2A), where the UVB component acted as a destabilizer. On the contrary, non-sterile glucose solution did not exhibit any UV absorption and, consequently,

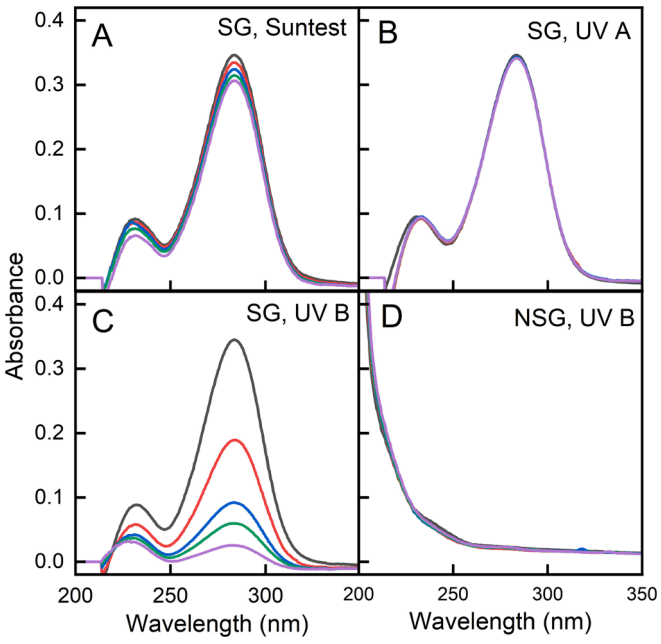

Fig. 2. UV spectra of irradiated samples of sterile glucose (SG) solution with increasing light doses: simulated solar light (A); UVA light (B); UVB light (C). Non-sterile glucose absorption spectra under UVB light increasing doses are reported as a reference (D).

no change in the UV spectrum under irradiation was detected (Fig. 2D).

The generation of singlet oxygen by a sterile glucose solution was measured with a simple and reliable spectrophotometric test (Kraljić and Mohsni, 1978) under our light conditions. This assay measures the bleaching of p-nitroso dimethylaniline (RNO) at 440 nm when a photosensitizing compound forms singlet oxygen under the effect of light. The imidazole present in the reaction acts as intermediate, increasing the sensitivity of the test. Under increasing UVB light exposure, sterile glucose solution exhibited a significant production of singlet oxygen (Fig. 3, black squares), whose formation was undetectable in non-sterile and saline solutions (Fig. 3, red circles, blue triangles, respectively). As supported by Fig. 1S, the sterile glucose solution was able to produce singlet oxygen under increasing light doses. The effect of UVB alone (C) was higher than UVA (B). The low component of UVB in the simulated solar radiation absorbed by 5-HMF could explain the formation of this reactive oxygen species in sterile glucose under the effect of the artificial sunlight (A).

4. Sterile and not sterile glucose effect on the conformation of Nivolumab under light

Fluorescence emission measurements were performed on formulated Nivolumab samples diluted with the three different solutions and the variation of fluorescence intensity was evaluated, since it sensitively reflects changes in the local microenvironment of aromatic residues (Lakowicz, 2002, 2006). The spectra obtained in the dark (Fig. 4, black lines) have a similar shape and the maximum wavelength of emission, after excitation at 280 nm, was ~ 350 nm suggesting a similar exposure to solvent of protein in the three different conditions. Under the standard dose of 720 kJ/m^2 of light, the variation of fluorescence emission intensity was negligible in samples dissolved in saline and NSG solutions and the curves appeared almost overlapped (Fig. 4A, B). On the contrary, a significant decrease of fluorescence intensity appeared when the mAb was diluted in sterile glucose (Fig. 4C, red line). By using a much higher dose of artificial sunlight (3360 KJ/m^2), all the three spectra changed suggesting that the high dose of light was able to affect the mAb conformation (Fig. 4A-C). However, the loss of fluorescence was considerable especially when the samples was dissolved in sterile glucose (Fig. 4C, orange line).

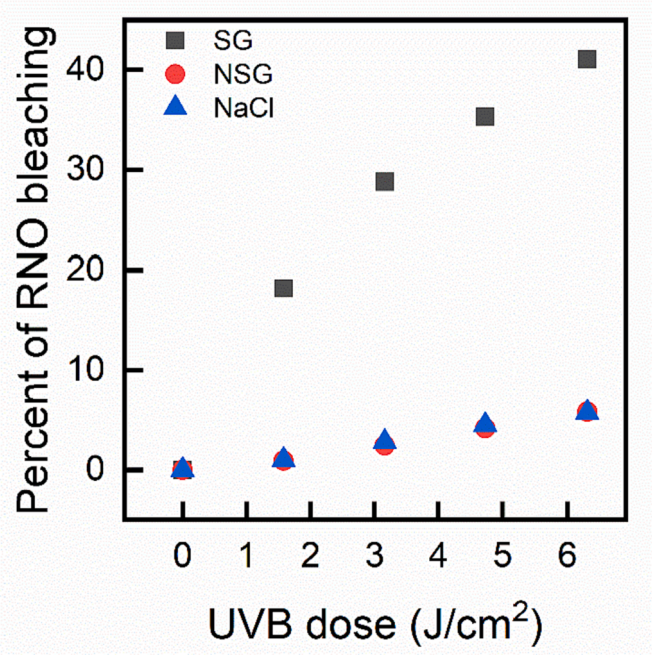

Fig. 3. Percentage of RNO bleaching as an indicator of singlet oxygen production under increasing doses of light by sterile glucose (black squares), non-sterile glucose (red circles) and saline solution (blue triangles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the dark as expected, 5-HMF (22.6 μg) added to the saline solution had no effect on protein fluorescence (Fig. 4D). On the contrary, when Nivolumab was diluted in NaCl added with 5-HMF and was then illuminated with 720 KJ/m^2 of light, a slight reduction of fluorescence was detectable comparable to the sample diluted in SG (Fig. 4E). The effect was marked under 3360 KJ/m^2 light dose (Fig. 4E, light blue continuous line, orange dashed line).

To compare the effect of the light exposure on the conformation of Nivolumab diluted in the three different solutions (NaCl, SG and NSG), circular dichroism (CD) spectra in the far (Fig. 5A-C) and in the near (Fig. 5D-F) UV range were also recorded. The samples were exposed to 3360 KJ/m^2 in the presence or absence of 5-HMF and the non-irradiated samples were reported as a control. In the far-UV CD spectrum performed on the control sample kept in the dark two minima were visible, one at ~ 218 nm distinctive of β-sheet secondary structure and another one at ~ 230 nm, according with the typical profile previously reported for Nivolumab (Torrente-López et al., 2022). The shape and the intensity of the CD spectra in the far UV barely changed under the different analyzed conditions of light and buffers, indicating that the secondary structure was scarcely affected by light and 5-HMF. The overall tertiary structure of irradiated Nivolumab, evaluated by near-UV CD, seemed conserved compared to the dark sample. Moreover, second-derivative UV spectra were calculated to better characterize the tertiary structure of Nivolumab under the light stress (Fig. 5G-I) and they showed superimposable profiles, thus confirming limited structural changes in the stressed mAb.

4.1. Physical modifications of Nivolumab under light stressor

mAb aggregation during drug handling is a widely reported phenomenon and, in fact, aggregate levels in biopharmaceuticals and final drug product are a key factor when assessing quality attributes of the protein (Carpenter et al., 1999). Several types of aggregates can form in protein solutions upon stress (Cromwell et al., 2006); as differences in the biological activity of aggregated species in respect to the monomeric ones can be expected, their presence must be evaluated. Here, the extent of soluble aggregates was analyzed by using size exclusion chromatography (SEC), (Fig. 6), and DLS measurements (Fig. 7). Moreover, a comparison between the samples dissolved in the different diluents and upon stress has been also done by SDS-PAGE (Fig. 7B). Although no visible aggregates were present, to remove possible insoluble particles from the mixture samples were filtered before loading onto the SEC column. The chromatographic analysis of formulated Nivolumab before light treatment showed a main peak at 2.87 mL elution volume corresponding to a value of ~ 140 kDa and hence to monomeric protein (Fig. 6). In the dark, similar behavior was detected for Nivolumab diluted in saline solution (Fig. 6, A), non-sterile glucose (Fig. 6, D) and saline (Fig. 6, G). Upon irradiation (3360 KJ/m^2), the intensity of the peak corresponding to monomeric species decreased and new peaks with shorter elution volume (2–2.5 mL) appeared (Fig. 6, B, E, H) in all the three diluents likely compatible with dimer and other high molecular species, although not completely solved. Moreover, 5-HMF, added to the three samples before irradiation, gave rise to the very intense peak eluting late in the chromatograms (Fig. 6 C, F, I).

Sample aggregation was determined in Nivolumab diluted with sterile glucose solution in a higher extent than in non-sterile or in saline solution after irradiation (Fig. 7A, red columns), but a notable increase in the protein aggregation was observed when samples were irradiated in the presence of 5-HMF (Fig. 7A, blue columns). The relative abundance of the soluble aggregates in the irradiated samples was calculated being 2.7 ± 2.0, 4.7 ± 2.6, 10.8 ± 5.7 % respectively for NaCl, NSG and SG dilution and, notably, 58.4 ± 2.1, 58.9 ± 2.7, 52.6 ± 5.7 %, in the

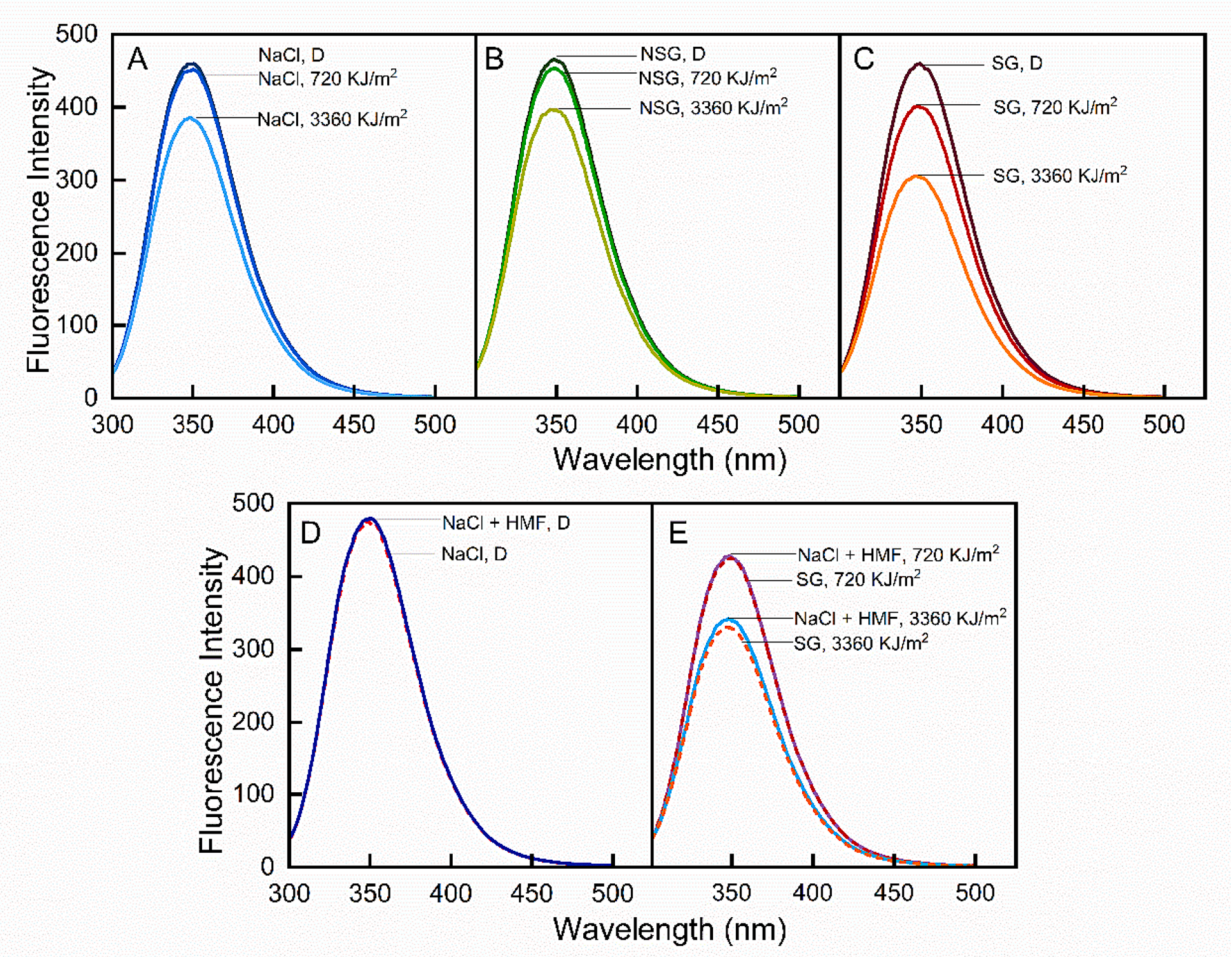

Fig. 4. Characterization of the effect of the light exposure on the structure of Nivolumab evaluated by intrinsic fluorescence spectroscopy. The measurements were carried out after exposure of the protein samples under 720 and 3360 kJ/m^2 of artificial sunlight in comparison with dark conditions (indicated with D). The samples were measured in 0.9 % saline (A), 5 % not sterile glucose (B), and 5 % sterile glucose (C) dilution. The effect of the addition of 22.6 μg 5-HMF (D, E) was also reported. Fluorescence emission spectra were collected after excitation at 280 nm.

case of the presence of HMF (Fig. 7A).

To better compare the aggregation state of Nivolumab in the different diluents, in the presence, and in the absence of light, the samples were loaded into an electrophoretic gel under denaturing conditions (Fig. 7B, gels B1 and B2). Under the reduced condition, both the mAb dark control and the irradiated samples dissociated into light chains (~25 kDa) and heavy chains (~50 kDa). Both NaCl and SG samples (lane 1 gel B1 and gel B2, respectively) showed no aggregation in the dark, neither under the addition of 22.6 μg/mL of 5-HMF in NaCl (lane 7 gel B1) and in SG (lane 4 gel B2). In diluted NaCl and sterile glucose (720 and 3360 KJ/m^2, lanes 2–3 in gel B1 and 2–3 in gel B2, respectively), higher-order aggregates dissociated to light chains, heavy chains, and a few faint bands with MWs of 50–100 kDa or higher, whose amount increased with increased light intensities. Notably, aggregated species were particularly evident when the sterile glucose solution was used as diluent of the formulation (lane 3 in gel B2) instead of non-sterile glucose (lane 9 in gel B2). Moreover, the addition of 5-HMF in NaCl and SG samples (up to 22.6 μg/mL, lanes 8–9 in gel B1 and 5–6 in gel B2, respectively), demonstrated an enhancement of the aggregation of Nivolumab under light exposure (720 and 3360 KJ/m^2). It is remarkable the presence of different bands corresponding to various aggregates, probable with some fragmentations again more evident in the light exposed sample (3360 KJ/m^2) added with 22.6 μg/mL of 5-HMF (lane 6, B2). To highlight the effect of 5-HMF (Fig. 7B3), another SDS-PAGE was run, comparing Nivolumab in the dark (lane 1), irradiated with 3360 KJ/m^2 (lane 2), irradiated with 3360 KJ/m^2 in the presence of 5-HMF (22.6 μg/mL) (lane 3), and in the dark in the presence of 5-HMF (22.6 μg/mL) (lane 4). The extent of aggregates clearly increased with increased concentration of 5-HMF, providing convincing evidence of the relationship between protein oxidation and aggregation. Notably, the effect of 5-HMF on the mAb in the dark is absent (lane 4, B3).

Furthermore, the same samples were also analyzed by means of dynamic light scattering (DLS) to give an insight about the particle size distribution of the preparations and the grade of uniformity of the various distributions. Nivolumab was dissolved in the three different diluents, and three samples showed a similar particle size when measured without irradiation (Fig. 7C, black lines). The calculated diameters are 15.18 ± 1.39, 18.62 ± 0.70, 21.65 ± 1.68 nm, respectively for saline, NSG and SG solution. After irradiation (red lines), in the presence of 5-HMF (blue lines) a marked increase of the diameter of the analyzed species was calculated (41.64 ± 3.89, 34.30 ± 1.46, 35.81 ± 2.77 nm) for the three solutions. As a general observation, the PDI values were in the range of 0.1–0.3 suggesting a moderate polydispersity (Stetefeld et al., 2016). However, they increased for the samples dissolved in glucose (roughly 0.3 for NSG solution). These data were in accordance with Hauptmann et al., (2018) who showed the same effect for mAb samples treated with sucrose.

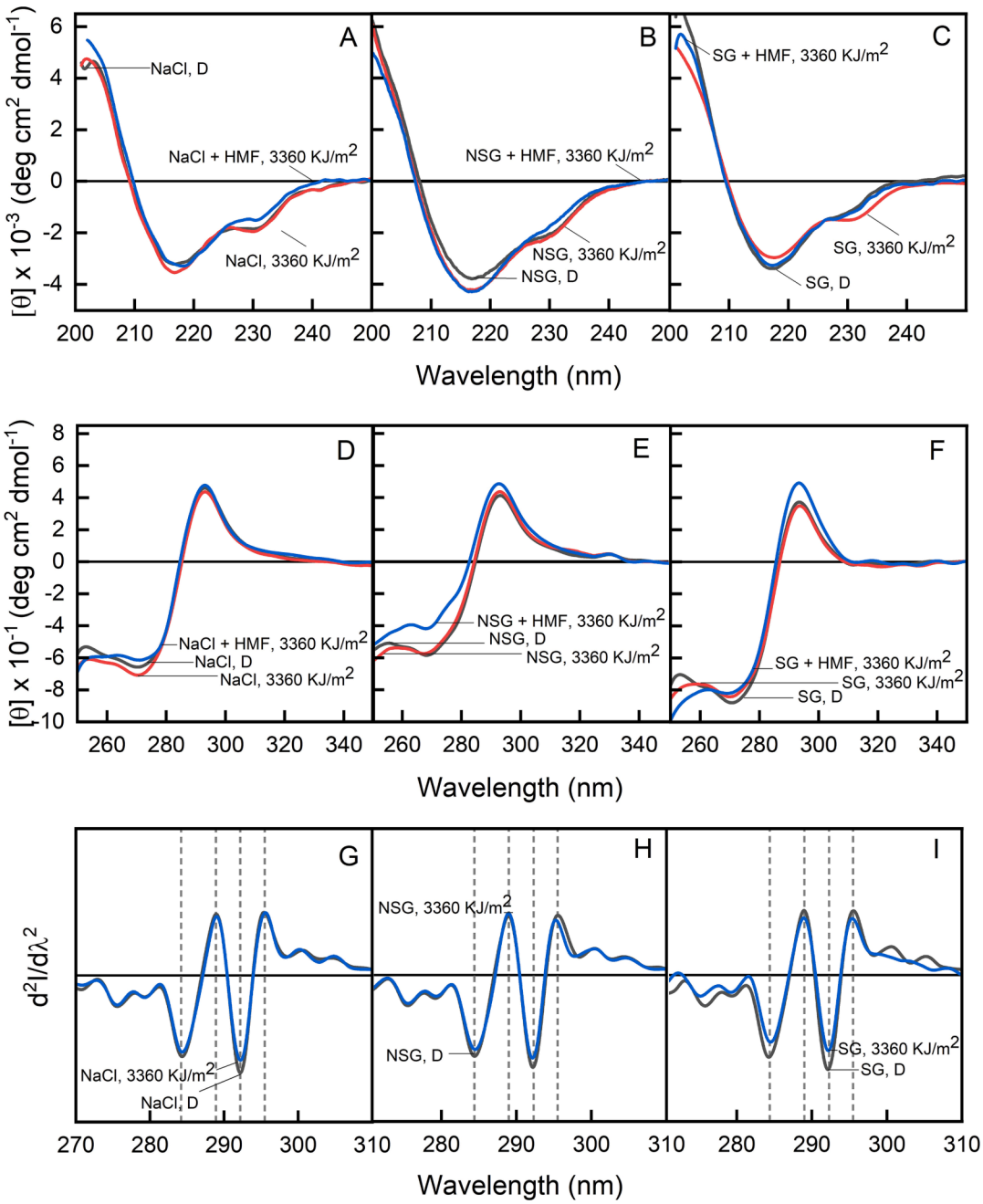

Fig. 5. Far (A-C), near (D-F) UV CD spectra and UV second derivative (G-I) of light exposed Nivolumab. The measurements were taken after exposure of the protein samples under 720 and 3360 kJ/m^2 of artificial sunlight. The samples were measured after dilution in 0.9 % saline (A, D), 5 % not sterile glucose dilution (B, E), 5 % glucose dilution (C, F), in comparison to the dark control (black).

4.2. Identification of the chemical modifications of Nivolumab by tryptic peptide mapping

A fingerprinting analysis by trypsin of Nivolumab was performed before and after irradiation (720–3360 kJ/m^2) in the three diluents. The proteolytic mixtures were analyzed by LC-MS/MS to verify the presence of chemical modifications in the side chains of residues in the polypeptide chain, matching the tryptic peptides with their calculated molecular mass. Overall, a good coverage with the mAb sequence was obtained (not shown). To evaluate a general tendency to chemical modification, we have analyzed peptides that satisfied some parameters: they should contain light-sensitive residues (especially Trp and Met); they encompass sequences belonging to variable regions of the light and heavy chains of the mAb; at least one should be inside the CDR. The selected peptides were L25-45, H44-57, H410-432, and H242-248. In Table 1, mass spectrometry results are summarized. In the dark, no (L25-45) or scarce (other peptides) Trp and Met oxidation were detected. Under the effect of light, as expected, the percentage of oxidation increased and particularly the formation of species containing Trp double oxidation was detected.

4.3. Biological activity of Nivolumab/Opdivo® diluted in SG after the light stressor

Nivolumab is a genetically engineered, fully human immunoglobulin (Ig) G4 directed against the negative immunoregulatory human cell surface receptor programmed death-1 (PD-1, PCD-1) with immune checkpoint inhibitory and antineoplastic activities. The Blockade

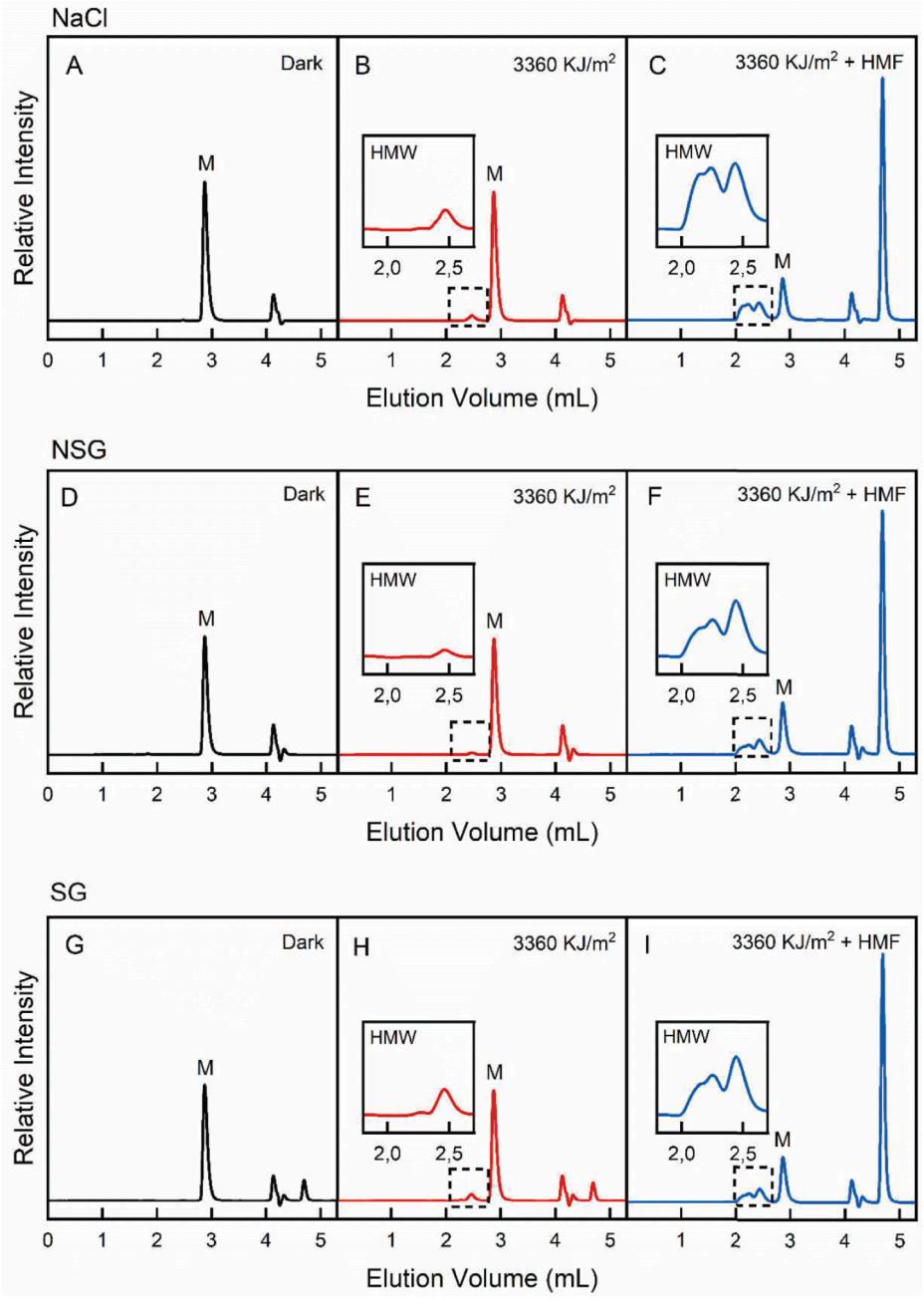

Fig. 6. SEC chromatograms of Nivolumab diluted in 0.9 % saline (A), 5 % not sterile glucose (B), and 5 % sterile glucose (C). Regions of the chromatogram corresponding to the aggregated forms are enlarged and highlighted. The chromatograms obtained in the dark (black line), under 3360 KJ/m^2 light (red line) and 3360 KJ/m^2 light and 5-HMF (blue line) are reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Bioassay was used for evaluating the biological activity of the formulated mAb as it reflects the mechanism of action of Nivolumab, and it can also give information on the residual specificity of the mAb toward its target PD-1 before and after the light stressors applied (dilution in sterile glucose and irradiation under artificial sunlight). For comparison, Opdivo samples diluted in SG were treated under pH 2.0 (Fig. 8C) used as forced denaturing condition control. A very small effect on Nivolumab biological activity was detected under both low and high light doses (720 and 3360 KJ/m^2, Fig. 8A-B, respectively), except a decrease of about 20–30 % under the acidic conditions with increasing mAb concentrations. This behavior seems in line with the preserved mAb conformation verified under the light stressor in SG.

5. Discussion

Light is a potential stress during the real life of a therapeutic mAb because it can affect the integrity and effectiveness of the drug (Le Basle et al., 2020). Oxidation is one of the main damages induced on proteins by light depending on the light source, intensity and wavelengths and giving rise to non-reducible cross-linked aggregates in the worse conditions (Pattison et al., 2012; Gupta et al., 2022). Here, we have studied the photostability of Nivolumab, the active principle of Opdivo®, for a better knowledge of its potential sensitivity to the light stressor. The novelty of this study is the impact of one of the main glucose degradation products, 5-HMF, on the photostability of the antibody under irradiation. Handling of mAbs in the hospital pharmacy and during their

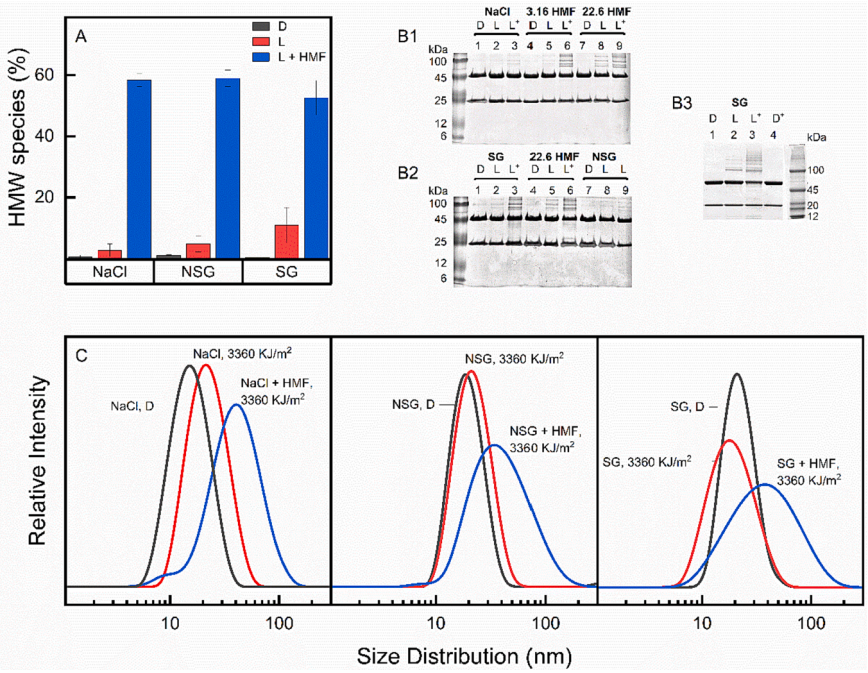

Fig. 7. Aggregation state probed by SEC (A), SDS-PAGE (B) and DLS (C). (A) Histograms representing the area under the SEC peak of the aggregated species of Nivolumab in 0.9 % saline, 5 % not sterile glucose, and 5 % sterile glucose solutions in the dark (black), after irradiation (red) and after the addition of 5-HMF (blue); (B) Samples loaded in SDS-PAGE (B1) are as follows: Nivolumab diluted in 0.9 % NaCl, Dark (1), 720 KJ/m^2 (2), 3360 KJ/m^2 (3), + 5-HMF (3.16 μg/mL) Dark (4), + HMF (3.16 μg/mL) + 720 KJ/m^2 (5), + HMF (3.16 μg/mL) + 3360 KJ/m^2 (6); + 5-HMF (22.6 μg/mL): Dark (7), + 5-HMF (22.6 μg/mL) + 720 KJ/m^2 (8), +5-HMF + 3360 KJ/m^2 (9). In the gel B2, the samples are as follows: Nivolumab diluted in sterile glucose, Dark (1), 720 KJ/m^2 (2), 3360 KJ/m^2 (3); + 5-HMF: Dark (4), + 5-HMF + 720 KJ/m^2 (5), + 5-HMF + 3360 KJ/m^2 (6); Nivolumab diluted in non-sterile glucose, Dark (7), 720 KJ/m^2 (8), 3360 KJ/m^2 (9). In the gel B3, a better resolution was obtained with a SDS PAGE gradient (4–12 %) for samples in 5 % SG: Dark (1), 3360 KJ/m^2 (2), + 5-HMF + 3360 KJ/m^2 (3), Dark 5-HMF (4). (C) DLS measurements of Nivolumab diluted in 0.9 % NaCl, 5 % not sterile glucose, and 5 % sterile glucose. The profiles obtained in the dark (black line), and under 3360 KJ/m^2 of light (red line) and 3360 KJ/m^2 of light and 5-HMF (blue line) are reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Analysis of the chemical modifications determined by fingerprinting analysis by mass spectrometry of Nivolumab under indicated conditions of dilution and light. The percentage of oxidation at the level of Trp and Met residues contained in selected peptides was reported. The symbol x 1 M and x 2 M indicates mono or double oxidation of Met residue, respectively. D and L refer to irradiated samples (L) and the control ones (D). 5-HMF was added at a final concentration of 22.6 μg/mL.

		W doubleoxidation L 25–45	W mono oxidation H 44–57	M mono oxidation H 410–432	H 242–248	
CTR		0	1.5	0	2.3	
D	NSG	0	0.6	0	2.2	
	SG	0	0	1.5	3.3	
	NSG + HMF	0	2.2	0.7	2.3	
L						
	NSG	0	0	2.9	6.9	
	SG	0.8	2.7	13.8	26.6	
	NGS + HMF	0	12.6	41.8	52.1 (x 1 M)	
1.4 (x 2 M)						

intravenous administration to the patient, that often takes some hours inside the hospital ward, exposes these sensitive molecules to different doses of light of different wavelengths, not so easily reproducible. Therefore, we primarily followed the basic guidance on the photostability testing of pharmaceuticals (ICH Q1/ICH, B10), designed to cover manufacturing and storage over shelf life, not covering the photostability of drugs during handling and administration. We started from the ICH light dose indications (720 kJ/m^2) for mimicking the real life of formulated Nivolumab and then we increased the light stressor conditions to get information on the chemical, physical and biological effects of different light exposures.

Nivolumab is a monoclonal antibody used in therapy for various types of cancer. It was approved in 2014 by the FDA for patients with advanced melanoma, and subsequently for various other carcinomas and oncological diseases (Rajan et al., 2016). Its dilution in sterile glucose or saline solutions represents a possible critical factor on the physico-chemical stability of the drug because the excipients with antioxidant and anti-aggregating properties are consequently diluted. Specifically, we have here proved that 5-HMF a harmful species for the oxidative damage to Nivolumab as it is able to form singlet oxygen, mostly under UVB irradiation. This is indeed the first demonstration of the oxidative damage induced by sterile glucose solution on a therapeutic mAb in combination with light.

As a matter of fact, the presence of GDPs in sterile glucose solutions, which are formed during the heat sterilization process are toxic *per se* (Bryland et al., 2010; Haybrard et al., 2017). Being a mixture of glyoxal, methylglyoxal, glucosone, 3-deoxyglucosone/3-deoxygalactosone, 3,4-dideoxyglucosone-3-ene, and 5-hydroxymethylfurfural, they are very reactive towards biological substrates (Leitzen et al., 2021). As an example, the reaction of formaldehyde with proteins can lead to the formation of methylol adducts on primary amino groups which can be partially dehydrated, yielding labile Schiff-bases. The latter can form cross-links with several amino acid residues, i.e., arginine, asparagine, glutamine, histidine, tryptophan, and tyrosine residues (Metz et al., 2004). Moreover, GDPs have shown to induce an oxidative damage to the diluted drugs after light exposure because of the production of

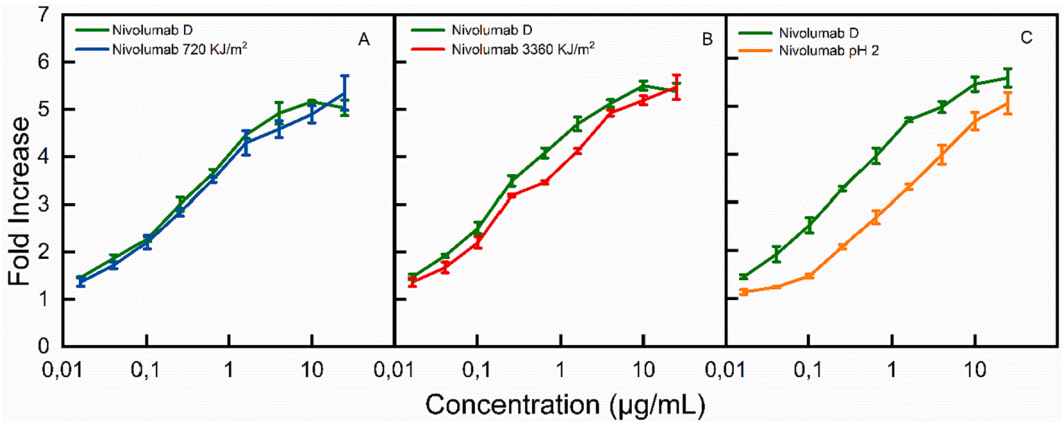

Fig. 8. Biological assay. PD-L1 aAPC/CHO-K1 and PD-1 Jurkat cells treated with serial dilutions of Nivolumab after exposure to 720 KJ/m^2 (A); 3360 Brusustung (B) of artificial sunlight; at low pH 2.0 (C) Dark samples are reported as a control.

singlet oxygen by 5-HMF triplet state (Brustugun et al., 2005). In our case, we have demonstrated that the presence of 5-HMF (3.16 µg/mL) in the sterile glucose solution used for diluting Opdivo® before patient administration is responsible for a pronounced oxidative effect on the mAb under irradiation, much higher than by the light alone. This result was confirmed when the same treatment, on saline or non-sterile glucose diluted mAb, induced much less extent of both oxidation and aggregation. Most likely, the aggregative process of Nivolumab depends on the combined effect of light, dilution, and the consequent oxidation of the Trp and Met side chains supporting a tight link between oxidative stress and protein aggregation processes (Lévy et al., 2019). Of course, dilution takes part on the extent of mAb degradation as the effect of protective excipients is reduced. However, the damage on this mAb appears more significant in the presence of 5-HMF in sterile glucose dilution, where consistent amount of singlet oxygen can form under light exposure. This finding adds new insights on the mechanism of formation of aggregates after an oxidative stress to mAbs recently attributed to free radical formation (Zheng et al., 2021). This oxidation tendency was intensely increased in the sample containing 22.6 µg/mL of 5-HMF, which is the maximum concentration allowed by the British Pharmacopoeia in injectable solutions.

By mass spectrometry analysis, we observed greater oxidation of some amino acids including Trp and Met, with both mono and double oxidations, compared to unexposed samples. Peptide fingerprinting identified the Trp residue in position 52 to be susceptible to double oxidation when Nivolumab was irradiated in the presence of sterile glucose. Noteworthy, Trp 52 belongs to the heavy chain CDR2 region (Fig. 1), and it is crucially involved in H-bond interactions with the N-loop of PD-1 (Tan et al., 2017). This result was not surprising as the complementarity determining regions compose flexible and solvent exposed regions in the mAbs. Nevertheless, the percentage of 44–57 oxidized peptide in Nivolumab did not drastically impact the structure of the mAb nor the binding activity toward its target PD-1, as shown by the conformational analyses and the biological activity assay. This result is, therefore, in line with all the conformational analyses carried out on the antibody before and after the light stress. Indeed, the tertiary structure of Nivolumab remained unchanged, both after irradiation with 720 kJ/m^2 and with higher doses of artificial sunlight, and even in sterile glucose or in the presence of 5-HMF added to the mAb dilutions. Similarly, the secondary structure was poorly influenced by light and 5-HMF.

Summing up, the physico-chemical stability of Nivolumab in samples unexposed to light could be higher to that reported in its Summary of Product Characteristic (SmPCs) ("Annex I Summary of Product Characteristics") and thus the therapeutic potential of this mAb could last for longer times than those declared on the leaflet included in the commercial formulation. Noteworthy, following irradiation extensive aggregation of the drug may occur, despite a slight effect on the overall structure. This phenomenon is expected to be relevant under dilution in sterile glucose and protection from light should be guaranteed in this condition. For this reason, some practical rules should be followed in all the stages of a mAb real life. Therefore, further study on the modifications of these drugs under the effect of light are needed as several differences concerning mAbs degradation are found depending on the mAb structure and the excipients present inside the formulation (Agarkhed et al., 2013; Allain et al., 2019; Sreedhara et al., 2016).

6. Conclusion

From our results, a great tendency towards aggregation of the antibody was detected when exposed to simulated sunlight and even more when it was diluted in sterile glucose. Fortunately, the destabilizing effect of light, in the presence and in the absence of glucose degradation products, did not appear to significantly affect the molecular target recognition of Nivolumab. The physical modifications of a therapeutic mAb should be taken into consideration as patients could experience complications during administration (Scheler et al., 2022) and more importantly the loss of efficacy and immunogenic side effects (Nabhan et al., 2020). Since sterile glucose is primarily used for pediatric therapies, special warnings and precautions for healthcare professionals should be considered to guarantee the conservation of the therapeutic properties and safety of the drugs in this diluent. From our experience, mAbs exhibit remarkable stability under various stress conditions. Their physico-chemical modifications mostly occur when they are exposed to severe and combined environmental circumstances. In perspective, our findings suggest to develop more tunable and specific instructions for their handling by healthcare personnel, based on the real life of each mAb.

CRediT authorship contribution statement

Elisabetta De Diana: Data curation, Formal analysis, Investigation, Visualization. **Elena Rizzotto:** Data curation, Formal analysis, Investigation. **Ilenia Inciardi:** Formal analysis. **Luca Menilli:** Data curation, Formal analysis, Investigation, Methodology, Validation. **Marina Coppola:** Validation. **Patrizia Polverino de Laureto:** Conceptualization, Methodology, Writing – review & editing, Resources, Project administration, Funding acquisition. **Giorgia Miolo:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The Authors enable the use of all the reasearch data

Acknowledgement

This work was supported by IMI Project: Real World Handling of Protein Drugs-Exploration, Evaluation and Education - RealHOPE No. 101007939.

We acknowledge the two master thesis students Sofia Troveretto and Nicolò Zangirolami for their help in performing some experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.123926.

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