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# Relationships between surface tensiometry properties and fluorescence intensity of dark and light exposed monoclonal antibody Nivolumab/ Opdivo® by using the contact angle method: A pilot study

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ABSTRACT

Monoclonal antibodies (mAbs) are a class of therapeutic proteins widely used for the treatment of different kinds of cancers and immune-mediated disorders.

During their real-life, they encounter various stressors, such as light exposure, able to modify their physico-chemical properties both in their formulation and when diluted for patient administration.

Several biochemical and biophysical analytical approaches are currently used to characterize the physico-chemical properties of mAbs, such as spectroscopic methods (i.e., UV absorption, fluorescence, near and far UV circular dichroism) for conformational studies, size exclusion chromatography, electrophoresis and dynamic light scattering for detecting aggregate formation, LC-MS for their chemical modifications. On these bases, our work is focused on the novel surface tension characterisation of one of these therapeutic mAbs, Nivolumab, in its formulation Opdivo® and after dilution and the relationship with classical fluorescence data. In particular, the mAb has been exposed to two different doses of simulated sunlight and the effect of the light stressor has been compared to the mAb kept in the dark. The application of Solid-like methodology, using the Rossi number as main surface tensiometry parameter, allowed us to demonstrate the close relationship between the physical, i.e., surface tension properties, and physico-chemical fluorescence emission of these big molecules.

*Abbreviations*

| | |
|---|---|
| $IAA_t^s$ | Integrated approach to study the bulk chemical composition (e.g., by spectroscopicmethods) bulk structure (e.g. by rheological measurements) and surface characteristics (e.g. by surface tensiometry) on the same material sample (s) and at the same time (t) |
| CA | Contact Angle (deg) |
| DC | Dispersion Component (mN/m) |
| mAb | Monoclonal antibody |
| PC | Polar Component (mN/m) |
| ST | Surface Tension of a liquid (mN/m) |
| PFPEd | Perfluoropolyether Drop |
| PFPEf | Perfluoropolyether liquid film "as solid substrate" |
| SCA | Static Contact Angle |
| SFE | Surface Free Energy ($mJ/m^2$) |
| SLM | Solid-like methodology |
| YL | Young-Laplace |
| $\chi^L_{PFPEf}$ | Rossi number (SLM) |

## Introduction

Monoclonal antibodies (mAb) are an important class of protein drugs widely used in pharmacology, clinical trials, and preclinical development stages. mAbs are modified sequences from IgG isoforms and can have different intrinsic structural stabilities [1]. The increasing prevalence of therapeutic mAbs, bispecific Abs, and antibody-drug conjugates has led to an increased need to evaluate and predict their stability during their real-life steps. The main analytical approaches currently used for the characterisation of mAbs consider their crystalline structure by

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X-Ray Diffraction (XRD), cryo electron microscopy (cryo-EM), and nuclear magnetic resonance (NMR) analytical methods. The Fourier transform infrared (FTIR) spectroscopy is used to characterise the secondary structures and secondary structural changes of proteins to verify their instability, while spectroscopic ellipsometry (SE) measures their dielectric properties. The dielectric properties can measure the adsorbed mass of proteins on surfaces, while Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) allow us to obtain surface images [1]. In the last years, the effect of different stressors on the stability of mAbs has been studied to know the weak points of their structural modifications, which can affect their efficacy and safety [2].

This issue is particularly relevant during their real-life phases, such as transport, storage, handling and administration [3,4].

Indeed, vibrations, temperature fluctuations, humidity, and light exposure [5] could induce chemico-physical modifications such as amino acid oxidations, deamidations, and aggregate formation, able to make the drug less active and in some cases immunogenic [6].

Multiple analytical methods are used for physico-chemical stability studies of mAbs: aggregation, fragmentation, and primary, secondary, and tertiary structure modifications can be detected through spectroscopic techniques (UV–Vis, Near and Far-UV Circular Dichroism, second derivative UV–Vis spectroscopy, intrinsic fluorescence spectroscopy). Specifically, LC-MS spectroscopy can identify amino acid modifications while separative methods (size exclusion chromatography or native or PAGE gel electrophoresis) give insights into the aggregation process and fragmentation, with DLS and FMI measurements applied for a deeper knowledge of the size of the aggregates formed.

From a surface point of view, Oom et al. investigated the interfacial phenomena at liquid/solid interfaces of mAbs [7], demonstrating that different antibody solutions have diverse hydrophobicity, self-association behaviour and interaction with various kinds of solid surfaces such as polystyrene and Teflon concerning their mass and viscoelastic properties.

From the liquid/vapour interface (l/v) viewpoint, the mAbs adsorb spontaneously, undergoing interfacial stresses which often cause aggregation phenomena. Interfacial stresses can cause destabilisation of therapeutic formulations of mAbs as they are the result of adsorption and aggregation at the liquid/vapour (l/v) interface.

As an example, at water/liquid surface an intermittent adsorption of hydrophobic (Phe, Tyr, and Ile) and polar (Thr, Ser, and Asn) folded mAbs residues present in the Fab domain occur at the water surface, depending on the surface tension ($\gamma_w$) of the water/vapor interface as demonstrated by Saurabh et al. using water simulations such as TIP4P-2005 and TIPs3P [8]. Saurabh et al. underlined also that the strength of water−protein interactions seems to have a significant impact on adsorption demonstrating its link with folded mAbs stability at water/vapour interface [8].

Furthermore, to increase protein stability, surfactants, such as polysorbate 20 (PS20), are added to mAb formulations to minimise interfacial adsorption phenomena [9]. In fact, in the presence of surfactants able to compete at the interface, the number of soluble aggregates (size $<$ 100 nm) depends on the amount of mAb adsorbed. Therefore, the number of insoluble aggregates (size $>$ 100 nm) does not depend on the surface concentration, but rather on the ability of the adsorbed mAb to interact and form a cohesive network [10,11]. Anyway, the difficulties related to the integration of data from different analytical techniques can be overcome using molecular modelling, providing further supporting information regarding the adsorption phenomena. For example, molecular dynamics and Monte Carlo simulations have gained a lot of importance in the study of protein adsorption [1].

However, the use of computer modelling in the study of mAbs interface processes is currently limited and needs to be integrated with experimental studies. This integration could improve the quality of protein adsorption predictions and expand the design of more stable mAbs [1]. As an example, the combined investigation of interfacial rheology, surface rheology, capillary viscometry, and neutron reflectometry performed on a model mAb solution with and without PS20, proved that the surfactant prevents mAb from adsorption [9]. As regards the surface tensiometry analytical technique, using the contact angle method, the selective extraction of mAbs from a culture broth was studied using a two-phase aqueous system (called ATPS) [12].

Furthermore, a recent study demonstrated the existence of a structure-surface relationship in three kinds of ointments, thus confirming the validity of the Integrated Analytical Approach applied to the study of complex matrices in pharmaceutical technological fields [13].

Based on this evidence, our pilot study focuses on the surface tensiometry characterisation of dark and light exposed Nivolumab/Opdivo® formulations and the relationships between their surface and physico-chemical properties. Nivolumab is a genetically engineered mAb produced from mouse-derived ovarian cells, formulated into the drug Opdivo® and endowed with an anti-PD-1 function capable of activating immune cells to enhance the antitumor response [14].

The common intrinsic fluorescence analysis of Nivolumab may provide useful information regarding the environment of aromatic amino acids (mainly Trp), and possibly the chemical modification of Trp under the effect of light, mostly oxidation [4].

From a methodological viewpoint, our pilot study is built specifically on the application of the Solid-like methodology (SLM) to determine the surface tension of Opdivo® as such and after dilution with two co-solvents, 5 % glucose and 0.9 % sodium chloride solutions. Indeed, Nivolumab is administered intravenously and often diluted in 0.9 % NaCl, which adds electrolytes potentially influencing the conformational stability, solubility, and formation of non-native aggregates [15]. Another diluent, widely used, is a 5 % glucose solution for pediatric treatments, although this diluent can induce hyperglycemia in patients when some medications are used or rapid aggregation upon mixing with dextrose and human plasma in vitro has been demonstrated for trastuzumab and bevacizumab [16]. However, sugars are often used for mAb formulation [17] for increasing protein stability [4].

The instability of Nivolumab under light exposure has been recently studied, and the effect of different diluents (i.e., saline and glucose solutions) has been demonstrated to have a role in the extent of oxidative damage and aggregate formation. Indeed, under light exposure, the glucose degradation products (GDPs) can generate ROS, which in turn increase the mAb modifications. Fluorescent spectra of dark and irradiated solutions showed a decrease of Trp fluorescence after irradiation, thus connecting these data with Trp oxidation and further aggregation [18].

Nevertheless, it's worth noting that the secondary and tertiary structure of the mAb seemed not to be affected by the chemico-physical modifications induced by light [18].

The effect of sugar molecules on the solution viscosity of mAbs has also been investigated through a dynamic light scattering method. Higher mAb solution viscosity is a result of the interactions between sugar and protein molecules in solution, in which exclusion of the sugar and hydration of the protein shell gives a more compact native state of the protein [19].

Different from Oom et al., we applied the Solid-like methodology (SLM) to determine the surface tension (ST) of untreated (dark) and light-treated mAbs formulation without the influence of friction forces and roughness surfaces. SLM is based on the ability of Fomblin HC/25®PFPE polyperfluoromethylisopropyl ether (now PFPE C-250®) to form a stable oleophobic, hydrophobic, and self-repellent (PFPEf) liquid film. This methodology demonstrates that PFPEf can be used as a liquid test for the determination of the surface tension characteristics of liquids [20,21]. The study of the relationships between ST and fluorescence properties of mAb samples was performed experimentally within the Integrated Analytical Approach ($IAA_t^s$) [22], defined as "an integrated approach to study the bulk chemical composition (e.g., by spectroscopic methods), bulk structure (e.g. by rheological measurements) and surface characteristics (e.g. by surface tensiometry) on the same material

sample and at the same time", using the single drop method in the CAs measurements performed on PFPEf because its homogeneity and reproducibility.

## Experimental

Nivolumab in the Opdivo® formulation (10 mg/mL concentrate for solution for infusion), Bristol-Myers Squibb Pharma EEIG (Plaza 254, Blanchardstown Corporate Park 2, Dublin 15, Ireland) was kindly provided by the Venetian Oncological Institute (IOV, Padova, IT). The excipients are 5.88 mg sodium citrate dihydrate, 2,92 mg sodium chloride, 30 mg mannitol (E421), 0.008 mg pentetic acid, 0.2 mg polysorbate 80 (E433), sodium hydroxide, hydrochloric acid, and water for injections [23].

### *Light exposure 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 (Table 1). Two doses of light shined on the samples simulated different times of sunlight exposure, i.e., 1.5 days (confirmatory test, ICH guidelines), 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: 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 suitable for being examined by the analytical techniques here used.

Irradiation. 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 10,460 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 1.5-day 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 were 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") [24].

### *Surface tensiometry*

The surface tensiometry analysis of dark and light treated mAb preparations was carried out at room temperature using a static tensiometer (optical goniometer) Drop Shape Analyzer 30 (Kruss Gmbh, Hamburg, Germany) equipped with a camera looking at a light source capable of displaying the real-time image of the drop shape formed when a liquid test comes into contact with the substrate (Fig. 1).

The surface tensiometry analysis was carried out using a DSA4 tensiometer (optical goniometer) (KRUSS, Hamburg, Germany) equipped with accessories and dedicated software.

**Table 1**
mAbs solutions tested.

| mAbs solutions ($N = 9$) | | | |
|---|---|---|---|
| Opdivo® | Dark | 720 KJ/m$^2$ | 10,460 KJ/m$^2$ |
| Nivolumab/NaCl | Dark | 720 KJ/m$^2$ | 10,460 KJ/m$^2$ |
| Nivolumab/glucose | Dark | 720 KJ/m$^2$ | 10,460 KJ/m$^2$ |

### *Static contact angle (SCA)*

The measurement of CA is possible because a drop of liquid, or liquid phase, deposited on a solid surface, or solid phase, assumes a well-defined drop shape which depends on the nature of the two phases when they come into contact with each other [25,26]. The CA measurement is based on image analysis of a drop of a liquid deposited on a substrate.

The real shape of the drop is studied based on the ideal one (reference), whose surface curvature depends on the balance between the surface tension (ST: mN/m) and the weight of the drop, on which the hydrostatic pressure depends. According to the Young-Laplace equation, there is a relationship between the radii of curvature $r_1$ and $r_2$, the ST and the Laplace pressure (p) according to Eq. (1).

$$\Delta p = \gamma_{f(l)v} * (1/r_1 + 1/r_2) \quad (1)$$

where $\Delta_p$ is the Laplace pressure, $\gamma_{f(l)v}$ is ST (mN/m) of the liquid, and $r_1$ and $r_2$ the radii of curvature of the drop.

The method is based on the goniometric measurement of the CA of a drop of liquid deposited on the surface of a solid substrate, both having proper properties of ST ($\gamma_L$) and SFE ($\gamma_S$) [25,26].

The analysis of the CAs in static conditions was performed after 0.5 s from the deposition of the liquid drop test on the substrate, upon reaching the equilibrium point between the liquid-vapour (l/v), solid-liquid (s/v) and solid-vapour (s/v) interfacial forces. For each mAb formulation, the "single drop" method was applied by measuring the relative contact angles (CA: deg) and corresponding surface tensiometry parameters because of the homogeneity of silica glass and PFPEf used as solid substrate.

The CAs values are expressed in radiant degrees (rad) and can be converted into surface tension of liquids and solids using conversion models such as Owens & Wendt-Rabel & Kaelbe (OWRK) [27–29].

The OWRK model is a system made up of two or more equations through which it is possible to correlate the degree of polarity of each test liquid to the relative CA measurement [25,26] according to Eq. (2):

$$\gamma s - \gamma l * \cos\phi = \gamma s - \gamma l - 2(\sqrt{\gamma^d}_s * (\sqrt{\gamma^d}_l) - 2(\sqrt{\gamma^p}_s * (\sqrt{\gamma^p}_l) \quad (2)$$

where $\gamma s$ is the SFE of the unknown substrate, $\gamma_l$ is the ST of the known test liquid, cos θ is the cosine of the CA measured in radian degrees, $\gamma_s^d$ is the DC of the unknown substrate, $\gamma_l^d$ is the DC of the liquid known test, $\gamma_s^p$ is the PC of the unknown substrate, and $\gamma_l^p$ is the PC of the known test liquid.

The OWRK model can also be used to evaluate the ST of unknown liquids using the CA method and test solids having known surface tensiometry properties. The evaluation of a liquid's ST using the CA method is possible thanks to the principle of permutability applied at surface tensiometry [20].

In this work, the characterization of the Surface Tension (ST: mN/m), Dispersion Component (DC: mN/m), and polar component (PC: mN/m) of dark and light-treated mAb preparations was performed using vitreous silica and a liquid film of PFPE as reference test solid and liquid perfluoropolyether "as solid substrate" by Solid-like methodology (SLM) [20,21].

*Solid like methodology (SLM).* The SLM is a procedure capable of determining the CAs at the interface between liquid samples and a liquid film of PFPEf intended as a "solid substrate" for surface tensiometry [20,21].

The SLM procedure provides a deposition of 0.5 ml of PFPE liquid drop (PFPEd) on a solid support. A homogeneous "liquid film of PFPE, spontaneously formed on the support, allows the measurement of CAs at interfaces between the PFPE liquid drop and the PFPE "liquid film"

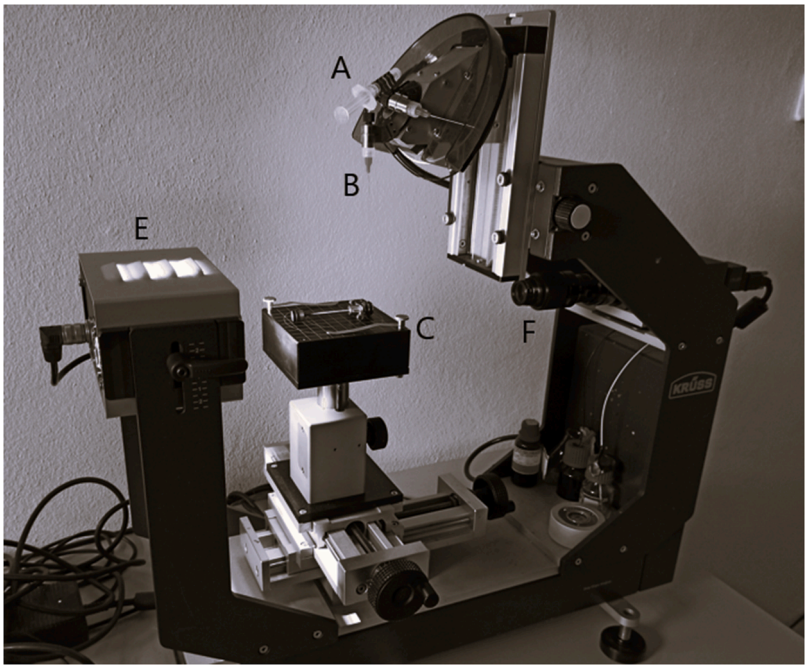


**Fig. 1.** Static tensiometer (optical goniometer) (A) syringe support, (B) syringe, (C) sample holder support, (E) light source, (F) camera.

(PFPEd/PFPEf), as well as between the PFPE "liquid film" and the drops of other test liquids [20,21]. The time required for measurements is a function of the quantity of PFPE "liquid film" deposited on the surface of the solid support. For example, a time of 60 s after application of 0.5 ml of PFPE drop on the solid support is needed to obtain a stable 1 mm thick "liquid film" [20,21]. The PFPE used for the development of SLM was Fomblin HC/25® PFPE (PFPEd) (Solvay Solexys, Milan, Italy), now commercialised with the name PFPE Oil C-250® (Fuzhou Topda New Material Co., Ltd, Fuzhou, China). Fomblin HC/25®PFPE (PFPEd), it is a polyperfluoromethylisopropyl ether (pertaining to the family of perfluoropolyether) having ST=18.1 mN/m, DC=18.0 mN/m, and PC=0.1 mN/m [30–35]. PFPEd is a transparent, colourless, odourless liquid polymer, permeable to vapour, insoluble in organic solvents, chemically and biologically inert, and non-flammable. PFPEd is a substance having hydrophobic, oleophobic, and "self-repellent" characteristics [20,21]. Specifically, PFPEd is used in the cosmetic field as an emollient, lubricating substance with moisture barrier properties [30,31].

PFPEd is used as a binding agent, plasticiser, and slow-release agent. More recently, the PFPEd fluoropolymer has been used for the first time both as a test liquid (PFPEd) for the determination of the SFE of natural and artificial solid (s) and semi-solid (ss) complex systems (e.g., skin, on topical formulations and geo-materials such as thermal mud) and as a "solid substrate" for the determination of the ST of liquids (PFPEf) [20,21].

The Solid-Like method (SLM) is applied to determine the Surface Tension (ST: mN/m), Dispersion Component (DC: mN/m) and Polar Component (PC: mN/m) of liquid systems after depositing 0.5 ml of PFPEf on an inert support [20,21] (Fig. 2)

Thanks to its characteristics, PFPE (f or d) can form a liquid film on the surface of the inert support (PFPEf). The SLM method is based on the possibility of determining the CA of an unknown liquid when it comes into contact with the PFPEf. The PFPEf is therefore considered as a "solid substrate" compatible with the surface tensiometry study of liquids [20,21]. SLM led to carrying out a surface tensiometry analysis of a generic liquid in a rapid, non-invasive manner without the influence of interfacial friction forces and surface roughness factors (r). The non-invasive characteristic of SLM, and surface tensiometry analytical technique in general, is because of the non-destructive approach to the analysis of complex systems. Different from qualitative-quantitative traditional approaches such as chemical and rheological investigations, SLM is

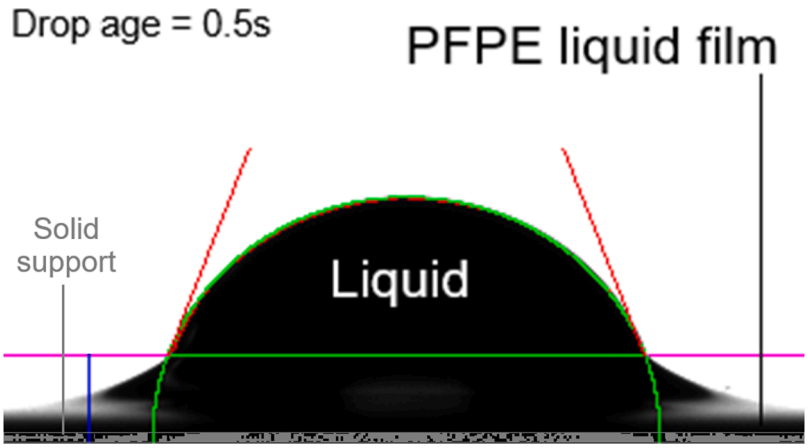


**Fig. 2.** Schematic view of Solid-Like methodology (SLM) and mAb solution drop profile on PFPEf liquid film.

based exclusively on the interfacial phenomena at SLM/liquid and liquid/vapour interfaces.

*The Rossi number ($\chi_L$PFPEf).* When a drop of unknown liquid comes in contact with the surface of the PFPEf, a characteristic meniscus is formed, caused by the absence of forces generated at the interface between the liquid and PFPEf (Fig. 3).

The 2D dimension of this PFPE meniscus depends on the surface tension characteristics of the unknown liquid. The surface tensiometer parameter that describes the size of the meniscus and its variations is defined as the Rossi number [20,21] (Eq. (3)).

$$c^{L}{}_{PFPEf} = m\left(CA^{M} - CA^{nM}\right) + b \tag{3}$$

where $CA^{M}$ is the contact angle of the generic liquid (L) measured on the liquid film by placing the baseline at its surface, $CA^{nM}$ is the contact angle measured by placing the baseline at the interface between the unknown liquid and the meniscus of PFPEf, m is the angular coefficient of the linear slope and b the intercept value.

The $\chi^{L}_{PFPEf}$ is a dimensionless pure number derived experimentally for the first time, developed in the surface tensiometry field for the characterisation of unknown liquids [20,21]. The $\chi^{L}_{PFPEf}$ can be directly extrapolated from the term ($CA^{M} - CA^{nM}$) using the linear plot $y$=mx+$b$. The $\chi^{L}_{PFPEf}$ changes as a consequence of the typical physico-chemical and surface tension property of each liquid analysed. The variation of $\chi^{L}_{PFPEf}$ of a liquid system can occur over time, also concerning the modification of its chemical and physical characteristics due to external treatments.

The absence of interactions between the liquid and the PFPEf is at the base of the $\chi^{L}_{PFPEf}$ because its variation depends exclusively on the physico-chemical properties, ST, DC, and PC, of the analysed liquid. Therefore, ST, DC, and PC values do not depend on the adhesion phenomena normally occurring at the interface between the analysed liquid and a solid substrate [20,21].

In our work, the CAs measured in static conditions were performed within the single drop method because of the homogeneity of PFPEf. Furthermore, the application of the SLM and the use of $\chi^{L}_{PFPEf}$ led to the determination of the Surface Tension (ST: mN/m, milli newton/meter), Dispersion Component (DC: mN/m, milli newton/meter), and the Polar Component (PC: mN/m, milli newton/meter) of the mAb Nivolumab.

*Contact angle (CA) measurements over time*

The CA measurements over time allow us to characterise the horizontal distension of a drop of liquid on the surface of a solid substrate by measuring the contact angles over time, 0.5 s after contact between the two phases [25,26]. This method makes it possible to evaluate the different surface tensiometry (adhesive) and chemical interactions that occur between two phases over time and to carry out many CA determinations ($12<N<2500$). In our work, the CAs measurements (300 frames) were performed for each dark and light-treated mAb formulation with a resolution of 2 frame/s, for a total time of 150,000 ms (150 s).

In the case of the SLM method, the measurements of CAs over time allow the vertical movement evaluation of the drop when it diffuses through the PFPEf liquid film and CAs are measured by Vertical Drop Contact Angle methodology (VDCA) [20,21].

The VDCA methodology led to determining the Vertical Drop Speed of a liquid drop when diffuses through the PFPEf liquid film (Eq. (4)).

$$VDS = dSA/dt \tag{4}$$

Where dSA is the variation of the Surface Area ($m^2$) at PFPEf/liquid interface over the time dt (ms or s).

In this case, 400 measurements (400 frames) of VDS were performed for a total of 300 analysis.

*Circle method.* The measurements of the CAs over time were performed using the Circle method because during the droplet distension over time, the CAs values tend to decrease under 30° [36]. The circle method is based on the circular arc equation applied to the shape of the optically determined droplet. The CA is then taken as the angle between the circular arc and the intersecting baseline, and then calculated as such. The measurement of the CA is less susceptible to interference from friction forces and surface roughness, for this reason the Circle method has been used for the determination of the contact angles over time as it allows to reduce of the distortions that occur during the measurements (flattening) by Young-Laplace method. The distortions in the CA measurement are also due to the high frequency of their determinations (2 frame/s), which tend to worst the influence of the surface roughness factor (r) and consequently of the CA measurement error (Fit error: μm).

Likewise, the Young-Laplace method for SCA [25,26], the CAs can be measured by placing the baseline at the interface between liquid and solid.

*Fluorescence analyses*

Fluorescence analysis was performed with a Varian Cary Eclipse spectrofluorometer. Emission spectra were recorded in the range 300 nm - 500 nm with the excitation wavelength of 280 nm, where aromatic amino acids, i.e., tryptophan and tyrosine, absorb before emission. Measurements were recorded with a scan speed of 120.00 nm/min in a quartz cuvette of 3 mL capacity and 1 cm path length. Baseline contribution was removed by its subtraction from the protein sample spectra. Measurements were conducted in triplicate. The instrument calibration is routinely performed according to the procedure provided by the manufacturer (Agilent, Santa Clara, CA).

While the irradiation of the samples named Opdivo, as such, 0.9 % NaCl and 5 % glucose, was performed at a concentration of 1 mg/mL, for fluorescence measurements, the concentration analysis used was 0,1 mg/mL, after diluting the samples with distilled water, NaCl and glucose solution, respectively.

Detailing these measurements, the presence of glucose degradation products, specifically 5-hydroxymethylfurfural, 5-HMF) able to absorb the excitation wavelength (280 nm) [37–39] was taken in consideration. Therefore, 3.16 μg/mL of 5-HMF (concentration present in commercial sterile glucose) was added to the three kinds of samples in order to level this interference during the fluorescence spectra recording.

*Statistical analysis*

The analyses of variance (ANOVA) were performed on surface

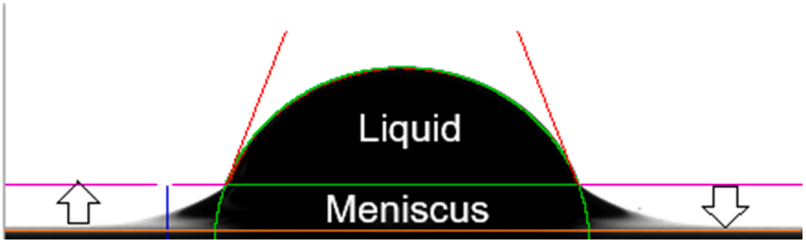


**Fig. 3.** Formation of a 2D PFPEf meniscus after the contact between the liquid and PFPEf.

tensiometry (SLM) and fluorescence (Intensity of fluorescence) data using Welch correction, O'Brien tests, and Wilcoxon methodology (SAS Institute Inc., Cary, NC, USA). An ANOVA test was performed to evaluate the discriminating capability of surface tensiometry and fluorescence analytical techniques.

The Welch correction compares two means to evaluate if they are equal, and it is an alternative to the classic ANOVA. The Welch correction can be used even if the data violates the assumption of homogeneity of variances [40,41].

The O'Brien procedure tests the assumption of homogeneity of variances because it is compatible with the analysis of variance (ANOVA) [42].

The Wilcoxon method (Wilcoxon signed-rank test) is a nonparametric test that evaluates whether the mean values of two dependent groups differ significantly from each other [43].

## Results

In Fig. 4, the correlation degrees between the fluorescence intensity measured on untreated Formulation A and the pH of the co-solvents are reported.

Fig. 4 demonstrates the high correlation degree ($R^2$~1) between the physico-chemical properties of NaCl 0.9 % and glucose co-solvents with the fluorescence intensity of Formulation A.

### *Untreated mAb solutions (dark)*

Fig. 5 reports the comparison between the variations of dark formulation A, B, and C contact angles measured at the interface with PFPEf ($CA^{nM}$) over time using VDCA methodology [15].

Fig. 5 shows that the polar nature of glucose and NaCl co-solvents produces a series of Formulation B and C $CA^{nM}$ values higher than Formulation A because of their different protein nature. In the condition of adhesion 0 (absence of interfacial interactions), the $CA^{nM}$ series depends only on the mAb formulation ST related to its physico-chemical nature.

The $CA^{nM}$ values measured at PFPEf/Formulation A, PFPEf/Formulation B, and PFPEf/Formulation C interface over time ($N = 1$, $N = 150$, $N = 300$), well correlated with the pH of the co-solvents (Fig. 6).

The shifts shown in Fig. 6 depend on the variation of the interfacial area measured over time and demonstrate the same high correlation degrees in relation to the different spreading drop speed data. This means that the pH of co-solvents is highly correlated with surface tensiometry parameter in all the steps considered ($N = 1$, 150, 300).

Furthermore, Fig. 6 confirms the strong effect of the two co-solvents concerning the correlation between pH and the $CA^{nM}$ measured ($N = 1$, 150, 300) at the interface between Formulation A, Formulation B, and Formulation C over time ($R^2$>90).

Fig. 7 shows the surface tensiometry models developed for the determination of the ST of the formulations by the Owens & Wendt model/Rossi number and the measurements of the CAs after the contact of PFPEf and glass, as reference test solid.

The Rossi number values of mAb (17.3), mAb/NaCl (21.0), and mAb/glucose (17.9) reported in Fig. 7 represent the real chemical, structural and surface tension properties of the three formulations. The differences between the Rossi number values have significance because this parameter is a pure number capable of amplifying the surface tensiometry differences of each liquid complex system in terms of CAs. As a consequence, the sensitivity of the $CA^{nM}$ measurement is higher than that measured on a solid substrate because of the absence of interfacial adhesion (adhesion 0), friction forces and surface roughness factor ($r = 0$) at folded Opdivo/PFPEf, mAb-glucose/PFPEf and mAb-NaCl interfaces. The higher value of the Rossi number measured at the interface between PFPEf and mAb-NaCl (21.0) seems linked to the increase of the aggregation process due to the presence of NaCl [44]. This is confirmed by the increase of DC (26.0 mN/m) of mAb in.NaCl, which is well correlated with the increase of Rossi number values (21.0) with a correlation degree of $R^2$=0.87.

Furthermore, the PC/DC values of mAb (2.36 mN/m), mAb-NaCl (2.01 mN/m), and mAb-glucose (2.73 mN/m) demonstrated a higher polarity of mAb in glucose cosolvent than mAb and mAb-NaCl. This appears following the hydrophilic properties of mAb (PC-DC=31.01 mN/m), mAb-NaCl (PC-DC=26.33 mN/m), and mAb-glucose (PC-DC=38.0 mN/m) [45]. Anyway, both PC-DC and Rossi number values appear linked to the aggregation of mAb in NaCl cosolvent, thus demonstrating its effect on the decrease of polarity (PC=52.26 mN/m) concerning mAb (PC=53.9) while the PC increases in mAb-glucose (59.82 mN/m).

Fig. 8 reports the relationship between ST of Opdivo® as such, mAb in 0.9 % NaCl and mAb in glucose and chemical parameters pH and osmolarity.

In Fig. 9 the linear relationship between $CA^{nM}$ and Fluorescence Intensity is reported.

### *Light treated mAb solutions*

In Table 1 the mAbs solutions treated with light and then characterised by both surface tensiometry approach and fluorescence are

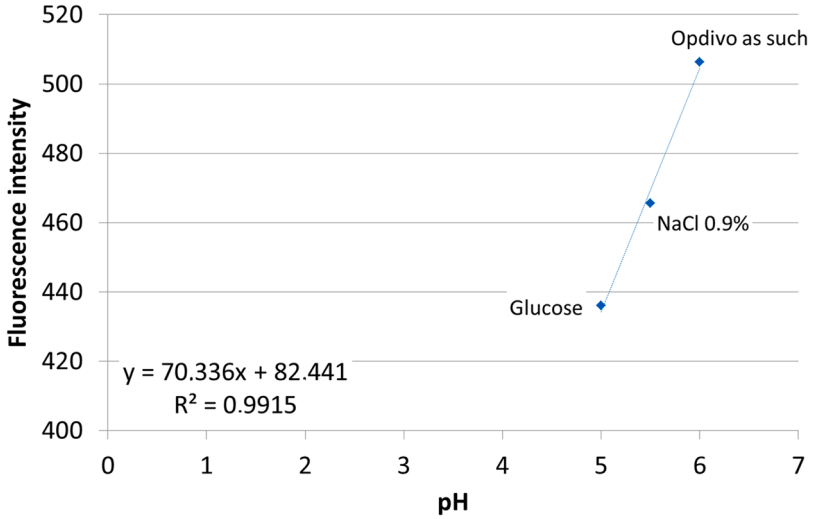


**Fig. 4.** Relationship between pH and fluorescence intensity measured on Opdivo®, in NaCl and glucose as co-solvents.

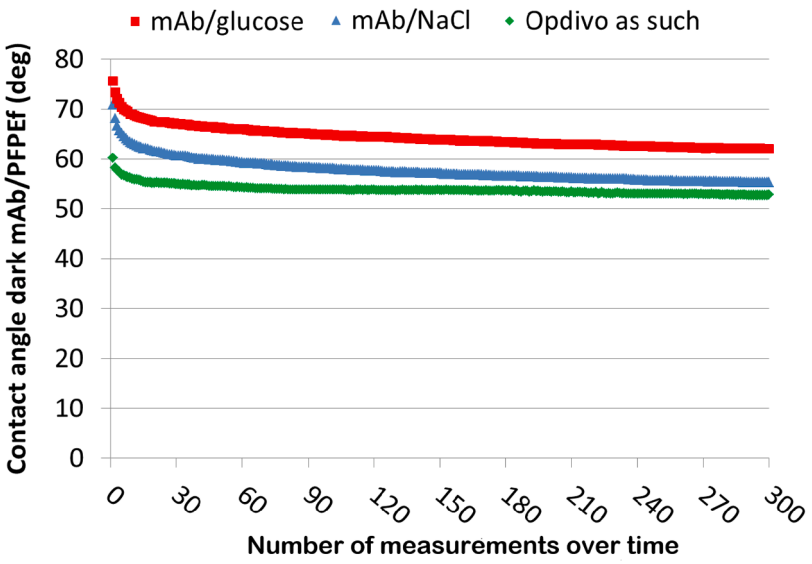


**Fig. 5.** Behaviour of contact angles ($CA^{nM}$) measured at PFPEf/Opdivo as such, PFPEf/Nivolumab in NaCl, and PFPEf/Nivolumab in glucose interfaces.

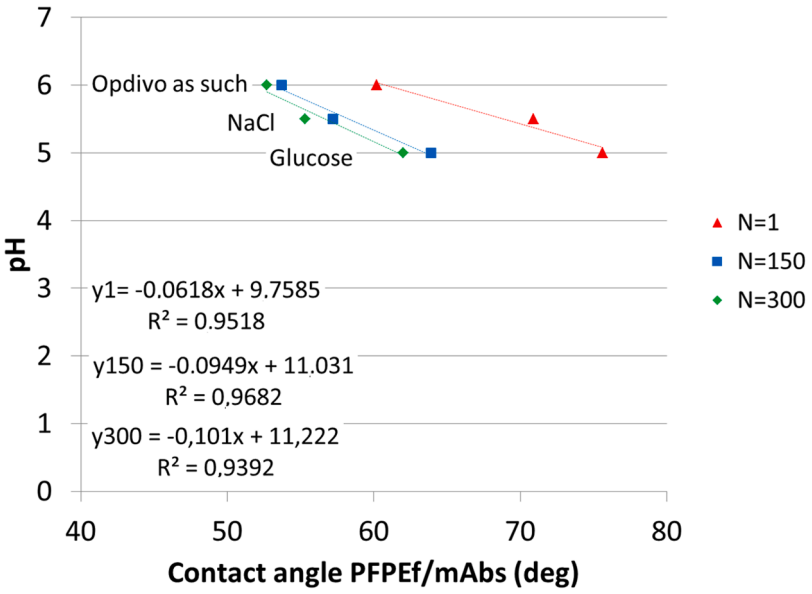


**Fig. 6.** Relationships between co-solvents pH and contact angle ($CA^{nM}$) measured at PFPEf/Opdivo as such, PFPEf/Nivolumab in NaCl, and PFPEf/Nivolumab in glucose interfaces after N1, N150, and N300 CAs measurements over time.

reported.

In Fig. 10a and b the comparison between Opdivo® dark solutions and after light treatment with 720 and 10,460 KJ/$m^2$ in 0.9 % NaCl and 5 % glucose, respectively, is reported.

The surface tensiometry data were obtained using the Vertical Dynamic Contact Angle (VDCA) methodology that expresses the Vertical Drop Speed (VDS: dSA/dt) of the drop after contact with PFPEf.

Fig. 10a and b demonstrated the high discriminating capability of surface tensiometry in the characterisation of different mAb formulations in relation to the light treatments after

In Fig. 11 it is reported a comparison between the VDS values of a drop of Opdivo® in 5 % glucose and in 0.9 % NaCl in function of the light treatments and in relation to the intensity of fluorescence (IF).

Fig. 11 shows the opposite behavior of VDS values between Opdivo® in 5 % glucose and 0.9 % NaCl in function of the light treatments at 720 KJ/$m^2$ and 10,460 KJ/$m^2$ in accordance with Fig. 10a and b. Fig. 11 reports also the correspondent values of intensity of fluorescence (IF).

Figures SM1a and SM1b report, respectively, the correlations between the intensity of fluorescence and light treatments concerning the solutions tested, and those between the intensity of fluorescence and $CA^{nM}$ concerning the light treatments.

N Material 1a

Supplementary Material 1b

SM 1a demonstrates satisfactory correlation degrees between intensity of fluorescence and light treatments concerning Opdivo® as such ($R^2$=0.99), in 0.9 % NaCl ($R^2$=0.99), and in 5 % glucose ($R^2$=0.99).

SM 1b shows satisfactory correlation degrees between intensity of fluorescence and surface tensiometry data expressed as $CA^{nM}$ (PFPEf/mAb solutions) in relation to dark ($R^2$=0.99), after light treatment with 720 KJ/$m^2$ ($R^2$=0.99), and with 10,460 KJ/$m^2$ ($R^2$=0.99). It is important to highlight that the Rossi number is directly derived from $CA^{nM}$, thus the variations appearing in SM 1b are different than those reported in Table 1.

In Table 2 a comparison between the Rossi number values and the

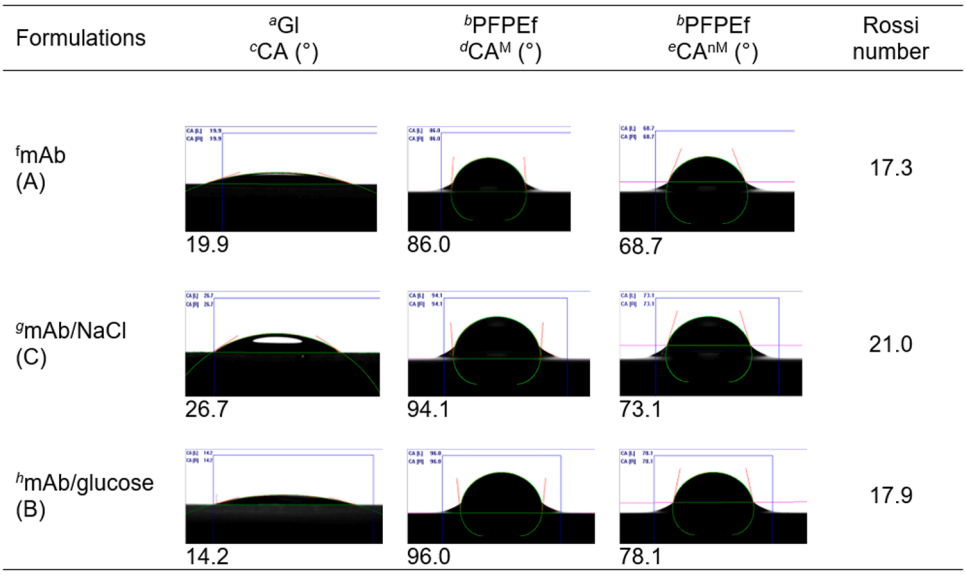


**Fig. 7.** Contact angle measurements at mAb/glass and folded mAb/PFPEf interfaces with Rossi number correspondent values, where a) is Glass, b) Perfluoromethyl isopropyl methyl Ether, c) Contact Angle, d) Meniscus Contact Angle, e) No Meniscus Contact Angle, f) Nivolumab/Opdivo® as such, g) Nivolumab/Opdivo® in 0.9 % NaCl, and h) Nivolumab/Opdivo® in 5 % Glucose.

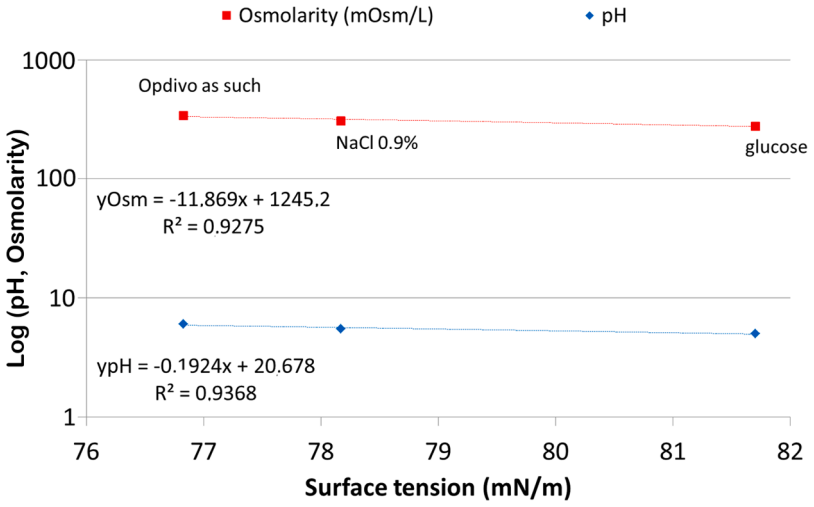


**Fig. 8.** Relationships between contact angles at PFPEf/mAbs interface ($CA^{nM}$) of Opdivo®, as such, Nivolumab/NaCl, Nivolumab/glucose and co-solvents osmolarity and pH.

corresponding $CA^{nM}$ measurements is reported for each formulation tested in dark conditions.

*Statistical analysis*

*Untreated mAb solutions*

The variance analysis of fluorescence data showed F report of 2.0052 and $p$=.1382 and demonstrated that the differences in intensities of fluorescence (IF) of Opdivo® as such, mAb in NaCl and mAb in glucose are not significant.

Differently, in the case of $CA^{nM}$ measured at the interface between PFPEf/Formulations, the tests O'Brien ($p$<.0001*) and Welch ($p$<.0001*) and the Wilcoxon method (Opdivo® as such vs mAb/NaCl $p$<.0001°, Opdivo® as such vs mAb/glucose $p$<.0001*, mAb/NaCl vs mAb/glucose $p$<.0001*) showed all different variances in the three formulations ($p$<.0001*).

*Treated mAb solutions*

The variance analysis of fluorescence data obtained from Nivolumab in 5 % glucose showed F report of 56.4310 and $p$<.0001. The p-values obtained from Opdivo® as such (dark), Nivolumab after treatment with 720 KJ/m$^2$ and that after 10,460 KJ/m$^2$ were <0.0001. This means that fluorescence analysis can characterise all mAb solutions in 5 % glucose.

As regards the analysis of fluorescence obtained from Nivolumab in 0.9 % NaCl, data showed F report of 2.8584 and $p$<.0581. In particular, the p-values obtained respectively from Opdivo® as such, Nivolumab after treatment with 720 KJ/m$^2$ and that after 10,460 KJ/m$^2$ were 0.0534 (dark vs Nivolumab after 10,460 KJ/m$^2$), 0.2260 (Nivolumab after 720 KJ/m$^2$ vs Nivolumab after 10,460 KJ/m$^2$), and 0.7773 (Dark vs Nivolumab after 720 KJ/m$^2$).

These results demonstrate that fluorescence analysis has a low capacity to characterise the mAb solutions in 0.9 % NaCl because the differences have no significance.

As regards the variance analysis of surface tensiometry data, represented by VDS data obtained from Nivolumab in 5 % glucose, it showed

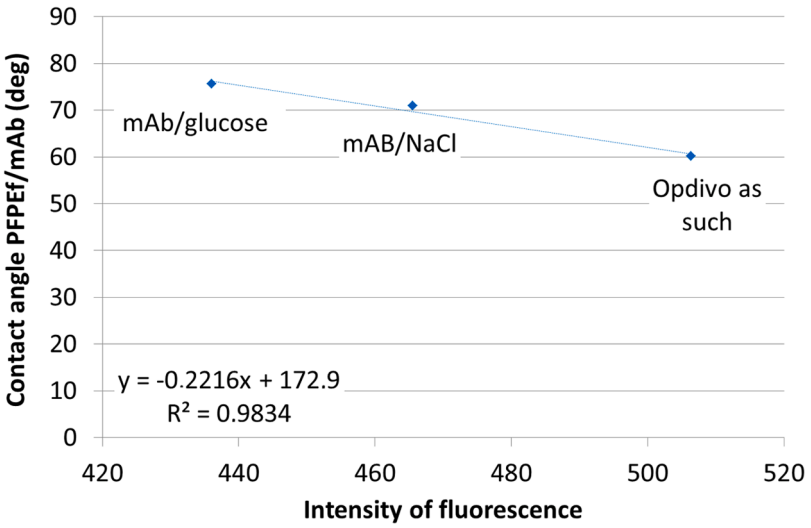


**Fig. 9.** Proportionally inverse relationship between surface tension (ST) and fluorescence intensity of Nivolumab/Opdivo® as such, in NaCl, and in glucose, diluted to 0.1 mg/ml with distilled water, NaCl and glucose solution, respectively.

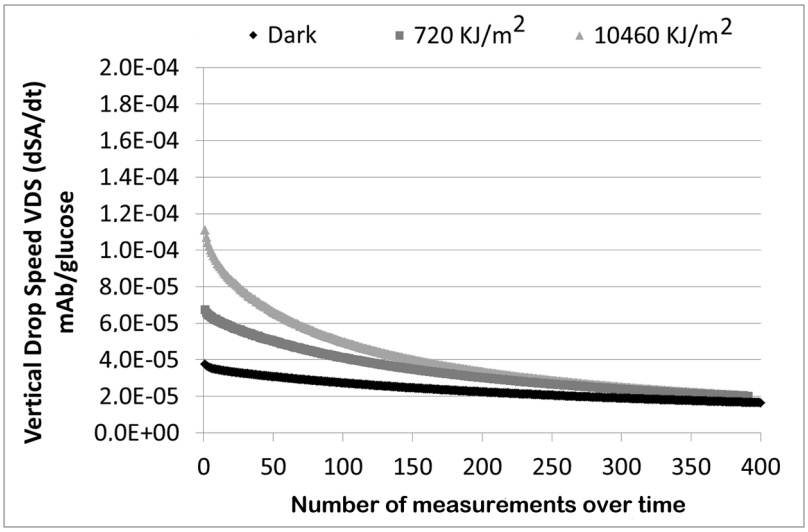


**Fig. 10a.** . Comparison between Vertical Drop Speed (VDS) of dark Opdivo® in glucose, after 720 KJ/m$^2$ and 10,460 KJ/m$^2$ light treatments.

F report of 1847.786 and $p<.0001$. The p-values obtained from Opdivo® as such (dark vs Nivolumab after 10,460 KJ/m$^2$ and dark vs Nivolumab after 720 KJ/m$^2$) and Nivolumab (Nivolumab after 720 KJ/m$^2$ vs Nivolumab after 10,460 KJ/m2) after treatments with 720 KJ/m$^2$ and 10,460 KJ/m$^2$ resulted of <0.0001. This means that surface tensiometry analysis can characterise all mAb solutions in 5 % glucose with the following fluorescence.

In the case of mAb solutions in 0.9 % NaCl, the variance analysis of surface tensiometry data showed F report of 281.7706 and $p<.0001$.

The p-values obtained from Opdivo® as such (dark vs Nivolumab after 10,460 KJ/m$^2$ and dark vs Nivolumab after 720 KJ/m$^2$) and Nivolumab (Nivolumab after 720 KJ/m$^2$ vs Nivolumab after 10,460 KJ/m$^2$) after treatments with 720 KJ/m$^2$ and 10,460 KJ/m$^2$ resulted of <0.0001 following surface tensiometry data. This means that surface tensiometry analysis is more capable of characterising all mAb solutions in 0.9 % NaCl than fluorescence because the differences have statistical significance.

## Discussion

The changes in fluorescence spectra of irradiated samples concerning those in the dark are most likely due to oxidation processes induced by light on aromatic amino acids, specifically on tryptophan. These mAb chemical side chain modifications can affect the physical properties of the overall system and therefore of its surface tension. Physical changes such as the formation of aggregates after light exposure could be responsible for the appearance of immunogenic side effects [46–56], lower therapeutic efficacy (lower drug concentration), and problems during administration (increase of viscosity during injection).

On this basis, tthe results obtained in our work highlight the strong link between physico-chemical characteristics of the three systems Nivolumab/Opdivo® as such (Formulation A), in 0.9 % NaCl (Formulation C), in 5 % glucose (Formulation B) and the intensity of fluorescence that depends on the co-solvents used. The evaluation of this link represents a fundamental premise for the study of the correlation between physico-chemical and surface tension data, in particular, the correlation between surface tension and fluorescence intensity.

From a surface tensiometry point of view, our work demonstrates that SLM is capable of discriminating the three mAb conditions exclusively based on their surface tension properties.

In this context, the relationship between fluorescence intensity/pH ($R^2 \sim 1$) (Fig. 4) and couples $CA^{nM}$/pH ($N = 1$; $R^2$=0.97, $N = 150$; $R^2$

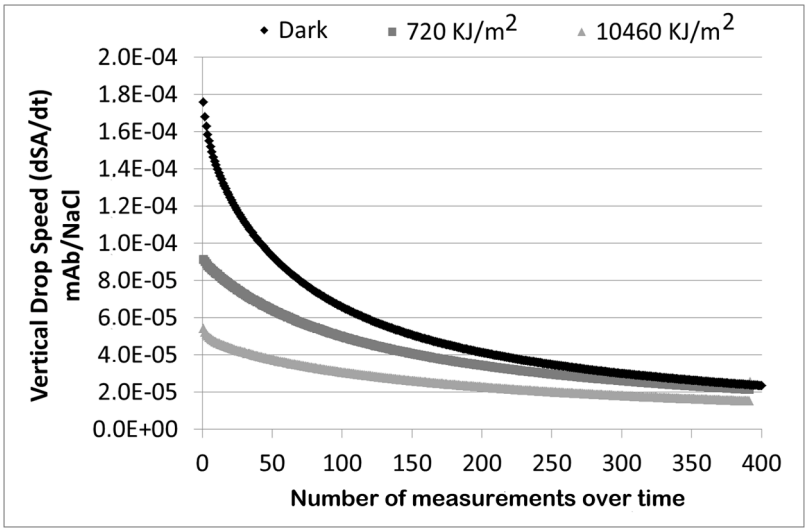


**Fig. 10b.** Comparison between Vertical Drop Speed (VDS) of dark Opdivo® in NaCl, after 720 KJ/m$^2$ and 10,460 KJ/m$^2$ light treatments.

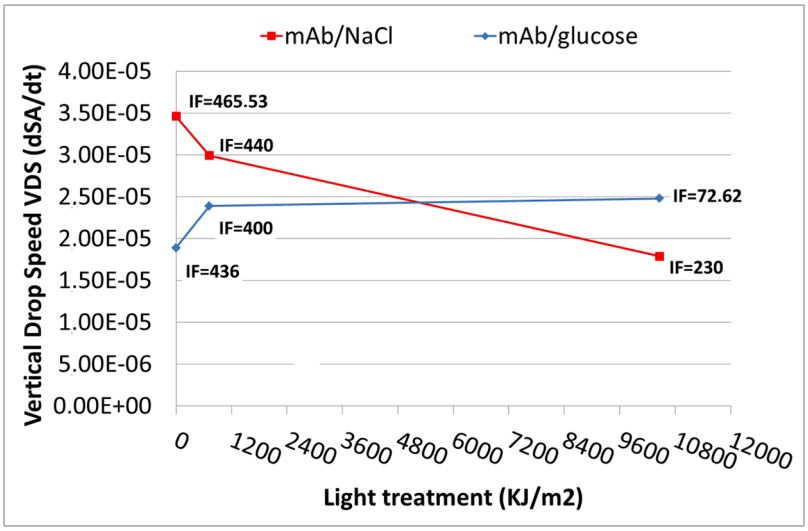


**Fig. 11.** Comparison between Vertical Drop Speed (VDS) values measured on droplets of Opdivo® in glucose and NaCl in function of the light treatments and intensity of fluorescence (IF).

**Table 2**
Comparison between the contact angle (CA$^{nM}$) measurements at folded mAb/PFPEf interfaces and Rossi number values.

| Formulations | [a]CA$^{nM}$ (°) | Rossi number |
|---|---|---|
| [b]mAb (A) | 68.7 | 17.3 |
| [c]mAb/NaCl (C) | 73.1 | 21.0 |
| [d]mAb/glucose (B) | 78.1 | 17.9 |

[a] No Meniscus Contact Angle.
[b] Nivolumab/Opdivo® as such.
[c] Nivolumab/Opdivo® in 0.9 % NaCl.
[d] Nivolumab/Opdivo® in 5 % Glucose.

0.94, $N = 300$; $R^2$ 0.95) data (Fig. 6), demonstrates the easy measurability of the effects of co-solvents and the influence of the mAb on the surface tension properties of the three formulation systems investigated.

The correlation analyses (Fig. 7) between surface tension and physico-chemical data (osmolarity; $R^2$=0.93, pH; $R^2$=0.94) confirm the measurability of the mAb effect on the systems with 5 % glucose and 0.9 % NaCl co-solvents also and, consequently, the influence of the amount of Nivolumab/glucose (Formulation B) on the correlation grade between the surface tension and intensity fluorescence data of Nivolumab/ Opdivo® as such (Formulation A) and Nivolumab/ NaCl (Formulation C) (Fig. 8).

As regards the mAb solutions treated with 720 KJ/m$^2$ and 10,460 KJ/m$^2$, the VDCA methodology puts in evidence the physico-chemical differences between Opdivo® solutions in glucose and NaCl due, respectively, to the effect of the molecular interactions between mAb and glucose and the influence of the ionic component on the surface tensiometry characteristics of both kinds of formulations.

These differences affect the behaviour of the surface tensiometry properties of Opdivo® formulations after light treatments at 720 KJ/m$^2$ and 10,460 KJ/m$^2$ concerning the different co-solvents used.

After the treatment with 720 KJ/m$^2$, the VDS values of Opdivo® in 5 % glucose increase slightly with respect to the formulation with 0.9 % NaCl, while the intensity of fluorescence decreases in both the formulations.

The increase of light treatment at 10,460 KJ/m$^2$ causes an inversion of VDS (Fig. 9a and b) and IF values (Fig. 10). This observation opens the study of the correlations between surface tensiometry and fluorescence and between light treatment and surface tensiometry.

Furthermore, the correlations between intensity of fluorescence and light treatments in relation to the solutions tested, and those between intensity of fluorescence and contact angle measured at PFPEf/solution interfaces demonstrate an indirect correlation between surface tensiometry data and light treatments for all the solutions tested (Fig. 11a, b).

The ANOVA test demonstrated that the Solid-like methodology (SLM), performed using a monolayer of PFPE liquid film ($p<.0001$*) is more capable of discriminating against the three formulations of mAb than fluorescence results ($p=.1382$).

Summarising, the SLM methodology is more capable of discriminating against untreated mAb solutions in NaCl than fluorescence, while in the case of glucose solution, the two techniques demonstrate to be comparable concerning the physico-chemical and surface tensiometry changes after light treatments. This phenomenon is since surface tensiometry measures the characteristics of the overall complex system. Consequently, the Rossi number and $CA^{nM}$ are very sensitive to the nature of co-solvents and their effects on protein stability in the dark and under the light treatments.

## Conclusions

The SLM method demonstrated a discriminating ability towards different Nivolumab/Opdivo® formulations (as such or diluted in NaCl and glucose) concerning the CAs measurements over time. This data highlighted the significant impact of the polar nature of the NaCl and glucose cosolvents on the contact angles of the different samples towards the PFPEf liquid film.

Moreover, the lack of adhesion (adhesion 0) to the mAb/PFPEf interface, due to the particular hydrophobic, oleophobic and self-repellent nature of the polyperfluoromethylisopropyl ether, and the imbalance between hydrophilicity and oleophilicity of the three formulations, enabled us to discriminate the polar effect due to the hydroxyl groups present in the glucose cosolvent from the ionic characteristic of the NaCl cosolvent, thus optimizing the surface tensiometry (surface tension) approach to the study of monoclonal antibodies by contact angle method.

The analysis of the fluorescence properties/surface tensiometry (surface tension) trend obtained by our sample's measurements confirmed the close relationship between surface tension and surface chemistry data, and the predominant effect of the cosolvent on the physical characteristics of the three formulations.

The close relationship between the fluorescence intensity and surface tension data demonstrates the influence of the matrix, recognised as "mAb-cosolvent" complex system, on the non-invasive and rapid detectability of the mAb.

The relationships between fluorescence and surface tensiometry data were confirmed by the comparison between both techniques applied on samples of mAb in glucose and NaCl after light treatment with 720 KJ/$m^2$ and 10,460 KJ/$m^2$. The results of ANOVA test highlighted a more discriminating capability of surface tensiometry for Nivolumab in both glucose and NaCl than the fluorescence intensity and demonstrates that the surface tensiometry analysis performed using Solid-like methodology (SLM) could be considered a new non-invasive and rapid tool to characterize different formulations of mAbs.

After mAb irradiation, some chemico-physical modifications occur in the protein structure. This variation can be visualized through the fluorescence emission of the mAb before and after the light exposure; at the same time, the same changes have an impact on the surface tensiometry properties of the mAb. The correlation analysis between surface tensiometry and fluorescence data suggests that the chemico-physical variation occurred in the mAb after irradiation affects the surface chemistry and therefore the contact angle values at the interface between PFPEf and the formulations tested. It is a matter of fact that the sensitivity of surface tensiometry approach depends on the absence of adhesion forces, friction forces and roughness at PFPEf/mAbs interfaces.

The results of this work open the potential use of surface tensiometry as a novel analytical technique capable of rapidly and non-invasively evaluating the surface chemistry modifications of "mAb-cosolvent" complex systems and the mAb stability during their handling.

In the end, our work promotes further investigations into the correlations between surface tension properties of mAb formulations and data obtained from multiple analytical methods for the detection of physico-chemical stability.

## 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.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.xphs.2025.103823.

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