Modulation of the Hydration Water Around Monoclonal Antibodies on Addition of Excipients Detected by Terahertz Time-Domain Spectroscopy

VINCENT P. WALLACE,[1] DENIS FERACHOU,[2] PENG KE,[3] KATIE DAY,[3] SHAHID UDDIN,[3] JOSE CASAS-FINET,[4] CHRISTOPHER F. VAN DER WALLE,[3] ROBERT J. FALCONER,[5] J. AXEL ZEITLER[2]

[1]School of Physics, University of Western Australia, Crawley, Western Australia 6009, Australia
[2]Department of Chemical Engineering & Biotechnology, University of Cambridge, Cambridge CB2 3RA, UK
[3]Formulation Sciences, MedImmune Ltd., Cambridge CB21 6GH, UK
[4]Analytical Biochemistry, MedImmune LLC, Gaithersburg, Maryland 20878
[5]Department of Chemical & Biological Engineering, ChELSI Institute, University of Sheffield, Sheffield S1 3JD, UK

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ABSTRACT: Terahertz time-domain spectroscopy (THz-TDS) has been shown to detect overlapping extended hydration layers around proteins. Here, we used THz-TDS to detect modulation of the extended hydration layer around monoclonal antibodies (mAbs) by the introduction of commonly used excipients. Proline and sucrose altered the hydration layer around a mAb (mAb1), which was observed as a negative shift in the plateau in absorbance above ~100 mg/mL mAb1 (~70,000 water molecules per mAb); arginine had no effect. At lower concentrations of ~10 mg/mL mAb1 (~700,000 water molecules per mAb) proline and arginine modulated the hydration layer, which was observed as a negative shift in the relative absorbance, whereas sucrose had no effect. The changes in the extended hydration layer were not translated to shifts in the thermal stability or protein:protein interaction parameter. The hydration layer of a second mAb (mAb2) was further shown to be modulated by more complex formulations composed of two or more excipients; although the differences in terahertz absorbance were not predictive of viscosity or long-term stability. THz-TDS promises to be a useful tool for understanding a protein's interaction with excipients in solution and the challenge will be to determine how to apply this knowledge to protein formulation.
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INTRODUCTION

Water is ubiquitous in the natural world but is often disregarded in studies on protein structure and function. Naturally occurring antibodies are surrounded by an assortment of water and cosolutes including small organic molecules, inorganic salts and macromolecules. The composition of the aqueous environment around an antibody is known to affect its structural stability[1–3] and solubility[4] and is a factor during binding interactions where water and cosolutes are displaced.[5] There are various theories that have been postulated to explain alteration of a protein's structural stability and solubility by excipients in terms of the modification of the hydration layer around the protein. 'Preferential interaction' explains alteration of the hydration layer in terms of waters displacement by the excipient[6,7]; 'preferential hydration' suggests additional water is introduced into the hydration layer because of the presence of the excipient in the bulk phase[8]; and 'competition for water' suggests the excipient's own hydration layer competes for water with the protein's hydration layer.[9,10] What has previously hampered research into the 'hydration layer' surrounding a protein and verification of the preferential interaction, preferential hydration and competition for water theories was the paucity of suitable analytical techniques. Terahertz time-domain spectroscopy (THz-TDS) has recently emerged as a suitable analytical technique for pharmaceutical[11–13] and biomedical[14–17] applications that is capable of interrogating the hydration layer and can be undertaken with bench-top laboratory instrumentation.

Information on the hydration layer is immediately relevant to protein formulation at high concentrations (>100 mg/mL) where it is known to play a role in (non-specific) protein–protein interactions; the understanding and control of which is critical to achieving high monoclonal antibody (mAb) concentrations. Currently, static and dynamic light scattering (DLS) measurements are available to quantify the osmotic second virial coefficient (B_{22}) and the related protein–protein interaction parameter (k_D), respectively, at low mAb concentrations in order to make predictions of the mAb's behaviour at high concentrations.[18] Net attractive protein–protein interactions may lead to aggregation and the generation of particulates.[19] An analytical technique probing the hydration layer would be complementary to existing techniques which directly probe the protein (or aggregate). Relating protein–protein interactions measured at low concentration to the hydration layer measured at high concentration may therefore give further insight to the colloidal stability, leading to a more rational formulation approach.

Correspondence to: Robert Falconer (Telephone: +44-114-2228253; Fax: +44-114-2227501; E-mail: r.j.falconer@sheffield.ac.uk)

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Terahertz time-domain spectroscopy uses electromagnetic radiation at frequencies between the far-infrared and microwaves. A lack of suitable light sources restricted research at the terahertz frequencies, in particular for strongly absorbing materials, until the late 1980s when new light sources were discovered.[20] Currently, the options for undertaking terahertz spectroscopy includes THz-TDS, synchrotron light sources, p-germanium lasers, free electron lasers and gas plasma light sources. Of these THz-TDS is the most versatile and practical for non-specialised analytical laboratories. THz-TDS was developed from the discovery that pulses of electromagnetic radiation at frequencies typically in the range of 0.1–4 THz (3–133 cm^{-1}) could be generated and detected using a photoconductive antenna. An ultrafast titanium:sapphire laser is used which generates femtosecond pulses of light that are focused onto a semiconductor material such as gallium arsenide which releases charge carriers which accelerate across an applied potential difference to generate a picosecond pulse of terahertz radiation. Both the amplitude and phase of the signal is then detected and converted from the time-domain to frequency-domain using a fast Fourier transform. This enables the frequency-dependent absorption coefficient to be calculated in the same manner as in infrared spectroscopy (FTIR).

The emergence of THz-TDS stimulated a lot of interest to investigate the properties of a wide range of materials[21–23] but it was not until 2000 when the first work was published on the use of THz-TDS to study proteins.[24] Since then, THz-TDS has been used to study a range of protein structures and dynamics which showed promise for smaller peptides, proteins and large macromolecular complexes.[25–28] What hindered analysis of proteins using THz-TDS was the strong absorption at terahertz frequencies of the highly abundant water compared with the small quantities of protein. In hydrogen bonding liquids, such as water, terahertz radiation is absorbed because of the strong interaction with the hydrogen-bonding network that has relaxation characteristics on the time scales of terahertz frequencies and is at an energy that matches its photon energy. The absorption of terahertz radiation by water is observed to increase with frequencies between 0.1 and 7 THz (3–200 cm^{-1}). At 1 THz, the absorption coefficient of water is 250 cm^{-1} at 20°C.[29]

Although water absorbance prevented analysis of protein structures in solution, it did enable the hydration layer around proteins to be studied. Simple experiments measuring absorption coefficient versus protein concentration demonstrated a non-linear relationship, suggesting water interacting with the protein surface had a different absorption coefficient to bulk (unperturbed) water; referring to this water as a hydration layer(s) implies their overlap as protein concentration increases above a certain point.[30–33] The unexpected finding was that the hydration layer around proteins was more complex and extensive than previously thought.[5] The original observation was made for a 79 amino acid fragment of lambda repressor protein using terahertz radiation generated by a p-germanium laser, and yielded an apparent hydration layer of approximately 20 Å thick.[30] A similar extended hydration layer was also observed around ubiquitin.[31] More recently, our research group has observed an extended hydration layer around synthetic peptides and human serum albumin using THz-TDS and synchrotron light sources, respectively.[32,33] Alternative experimental approaches such as extended frequency range depolarized light scattering[34] and ultrafast two-dimensional infrared spectroscopy[35] have observed similar complexity in the interaction between the water and a protein surface.

Here, we take the first experimental steps required to bridge the emerging THz-TDS technique to biopharmaceutically relevant formulations of mAbs. We also assess THz-TDS suitability for verifying the preferential interaction, preferential hydration and competition for water theories. The excipients we have chosen: sucrose, arginine and proline, are well established in the literature in the context of protein formulation on account of their distinct classification (non-reducing sugar, basic and neutral amino acid, respectively) and mechanisms of protein stabilisation.[3,36,37] We investigate the concentration-dependent effect of mAbs on the hydration layer and show how these effects are modulated by the addition of small molecule excipients and more complex formulation buffers. To determine whether the change in hydration layer was evident using techniques that are typically used in early stage protein formulation, we also measured the concentration dependent thermal stability of the mAb domains using differential scanning calorimetry (DSC) and the weak protein–protein interaction using DLS.

EXPERIMENTAL

The two different mAbs (termed mAb1 and mAb2) tested here were kindly provided by MedImmune, Granta Park, UK and are the IgG1 isotype. MAb1 and mAb2 have MWs of ~144 and 148 kDa, isoelectric points (pI) of 8.4 and 8.5 and mass extinction coefficients of 1.44 and 1.58 $(mg/mL)^{-1}$ cm^{-1}, respectively. MAb1 is relatively stable over a range of pH values and ionic strengths which have been described in detail previously;[18,38] this is in contrast to mAb2 which is less physically stable and required optimization of formulation conditions which are described below as Formulations A, B and C. Sucrose, L-arginine and L-proline were obtained from J.T. Baker, Center Valley, Pennsylvania, USA at pharmaceutical grade, and water was purified to 18.2 MΩ cm. MAb1 was first exhaustively dialysed using Slide-A-Lyzer dialysis cassettes (10k MWCO; Thermo Scientific, Paisley, UK) into 50 mM sodium acetate, 20 mM sodium chloride, pH 6.0 (hereafter termed 'acetate buffer'). Where excipient was added (200 mM of sucrose, arginine or proline), this step was made prior to final pH adjustment of the formulation buffer before dialysis. The mAb was then concentrated to ~200 mg/mL using an Amicon Ultra centrifugal filter (30k MWCO; Millipore, Nottingham, UK), with concentration calculated during centrifugation with reference to the extinction coefficient and absorption coefficient measurement at 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Paisley, UK). The concentrated solutions were filtered through a 0.45 μm membrane filter and stored for short periods of time (days) at 4°C–8°C before measurement by THz-TDS.

For mAb2, three different formulation buffers were prepared: Formulation A consisted of 20 mM histidine, 20 mM sodium chloride, 240 mM proline, pH 6.0; Formulation B, 20 mM succinate, 20 mM sodium chloride, 95 mM arginine, 180 mM mannitol, pH 6.0; and Formulation C, 20 mM succinate, 20 mM sodium chloride, 240 mM mannitol, pH 6.0. Dialysis, concentration and filtration were carried out for mAb2 in the same manner as described for mAb1, above. For each mAb in each buffer, a series of dilutions were made in the same buffer to get a range of concentrations between 1 and 150 mg/mL,