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Enabling quantification of protein concentration in human serum biopsies using attenuated total reflectance – Fourier transform infrared (ATR-FTIR) spectroscopy

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ABSTRACT

Changes in protein concentrations within human blood are used as an indicator for nutritional state, hydration and underlying illnesses. They are often measured at regular clinical appointments and the current analytical process can result in long waiting times for results and the need for return patient visits. Attenuated total reflectance – Fourier transform infrared (ATR-FTIR) spectroscopy has the ability to detect minor molecular differences, qualitatively and quantitatively, in biofluid samples, without extensive sample preparation. ATR-FTIR can return an analytical measurement almost instantaneously and therefore could be deemed as an ideal technique for monitoring molecular alterations in blood within the clinic.

To determine the suitability of using ATR-FTIR spectroscopy to enable protein quantification in a clinical setting, pooled human serum samples spiked with varying concentrations of human serum albumin (HSA) and immunoglobulin G (IgG) were analysed, before analysing patient clinical samples. Using a validated partial least squares method, the spiked samples (IgG) produced a linearity as high as 0.998 and a RMSEV of $0.49 \pm 0.05\,mg\,mL^{-1}$, with the patient samples producing R^2 values of 0.992 and a corresponding RMSEV of $0.66 \pm 0.05\,mg\,mL^{-1}$. This claim was validated using two blind testing models, leave one patient out cross validation and k-fold cross validation, achieving optimum linearity and RMSEV values of 0.934 and $1.99 \pm 0.79\,mg\,mL^{-1}$, respectively.

This demonstrates that ATR-FTIR is able to quantify protein within clinically relevant complex matrices and concentrations, such as serum samples, rapidly and with simple sample preparation. The ability to provide a quantification step, along with rapid disease classification, from a spectroscopic signature will aid clinical translation of vibrational spectroscopy to assist with problems currently faced with patient diagnostic pathways.

1. Introduction

The analysis of biofluids such as serum using vibrational spectroscopy is considered a potential solution to current problems with early and accurate diagnosis of many diseases [1] and promises improved patient mortality, morbidity and quality of life [2]. Biofluids are routinely obtained following a minimally invasive procedure, providing a large sample volume that contains biomolecular components such as proteins, amino-acids, lipids and carbohydrates in relative concentrations which are highly dependent on demographical characteristics and physiological or pathological status [3]. Clinicians establish a diagnosis from several criteria, including; medical history, clinical symptoms,

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imaging data and biological exploration. Numerous diseases are characterised by a qualitative or quantitative modification of a specific biological parameter, while others are associated with a biological signature, changes in multiple biological parameters [4,5].

Proteomics, peptidomics and metabolomics are often studied through nuclear magnetic resonance [6], mass spectrometry [7] or capillary electrophoresis [8]. A large number of proof-of-principle studies have identified diagnostic markers for cancers [9–12]. However, there is extensive sample preparation associated with these techniques. ATR-FTIR can provide a spectral profile of all the macromolecular classes contained within serum and a signature, as opposed to single markers, could be advantageous when analysing a heterogenous disease such as cancer. Vibrational spectroscopic investigations have resulted in a large number of proof of principle studies that show promising results [13].

The diagnosis of gliomas (high-grade and low-grade) from non-cancer through a combination of ATR-FTIR and multivariate support vector machine analysis (SVM), was achieved with average sensitivities and specificities of 93.75 and 96.53% respectively for human serum samples [14]. In 2016, a large serum study using FTIR spectroscopy was completed, reporting the discrimination of cancer vs non-cancer patients with a sensitivity of 91.5% and specificity of 83.0%, as well as deciphering cancer severity and the primary site of metastasis [15]. These classification values were then improved to 92.8 and 91.5%, sensitivity and specificity, by executing Random forest and 2D correlation analysis in combination [16]. The application of vibrational spectroscopy to analyse tissue sections, as well as single cells [17,[18], has also been hugely successful. The advantages of vibrational spectroscopy, such as ATR-FITR, and high classification values demonstrates a potential use as the gold standard for patient disease screening using serum [19–21].

To facilitate the translation of an infrared spectroscopy based diagnostic test, the incorporation of a quantification step could be regarded as beneficial and complementary to current clinical practice as the majority of clinical tests are currently based upon quantitative values as opposed to signatures or fingerprints. Protein vibrations are often the most prominent in a biological infrared spectrum [22]. Furthermore, protein concentrations are systematically measured in routine practice; they are useful to interpret biological parameters, discuss nutritional status, extracellular hydration status or to help in the diagnosis of some diseases. Specific proteins such as human serum albumin (HSA) and immunoglobulinG (IgG), (as well as the ratio of the two), may be altered in the case of inflammation, infection, unexplained weight loss, fatigue or act as symptoms of kidney or liver disease [23,24]. HSA constitutes between 57–71 % of the serum composition, and globulins 8–26 % [25]. HSA and IgG could be regarded as ideal to produce models in order to demonstrate an ATR-FTIR spectroscopic test capable of quantifying proteins.

Infrared spectroscopy enables the production of a unique spectrum representative of the fundamental molecular vibrations that occur within the sample, that provides a ‘fingerprint’ of the sample [26,27]. The combination of the rapid collection method obtained through the FTIR systems and spectroscopic method development has accelerated biomedical research using infrared spectroscopy. In particular, ATR-FTIR spectroscopy has been shown to be suitable for biological materials, due to the minimal sample preparation and the ability to analyse a variety of samples types, including serum [1,28–30]. An advantageous property of IR based techniques, is that they obey the principles of the Beer Lambert law [31], allowing quantification of a given molecule relative to the absorbance of light in the sample it is travelling through. This enables ATR-FTIR spectroscopy to quantify specific biomolecule concentrations, as the proportion of light absorbed by the sample will correlate with the concentration of molecules within a sample.

This is evident from the wide variety of research carried out, quantifying particular biomarkers from biofluid samples [32–34]. For example, the analysis of dried serum deposits using transmission spectroscopy highlighted the ability to quantify eight serum analytes [35] and the simultaneous quantification of glucose and urea analytes in addition to malaria parasitaemia from a single drop of blood dried on a glass slide [36]. The latter highlights the capability of using ATR-FTIR spectroscopy to determine disease and metabolic state, through the identification and quantification of chemical parameters associated with the disease diagnosis. Furthermore, the concentration of *in situ* DNA within cells [37], as well as the metabolite concentrations in urine [38] and saliva [39], could be determined using ATR-FTIR and bovine IgG was quantified using transmission and ATR-FTIR spectroscopy [40]. The quantification of glycine, a low molecular weight fraction (LMWF), provided evidence that ATR-FTIR spectroscopy can monitor systemic spectral modifications created by spiking human serum with lyophilised glycine [41]. Additionally, the removal of high molecular weight fractions (HMWF), through centrifugal filtration, led to an increased precision and accuracy of the quantitative models based on the partial least squares algorithm [42]. Research carried out by Perez-Guaita in 2012 [43], showed the possibility of determining total albumin, total globulin and immunoglobulin concentrations through the analysis of 50 µl liquid serum samples deposited on an ATR crystal cell. This work highlighted the potential for ATR-FTIR to act as a green alternative to current methods used within hospitals, through the removal of reagents and implementation of relatively cheap and simple instrumentation. However, no sample preparation study was performed in order to establish the optimum sample preparation with minute volumes of serum.

Infrared spectral datasets are information rich, highlighting underlying biological and structural differences. Coupled with powerful multivariate analysis approaches, they have the ability to differentiate between disease classes by extracting relevant information. Multiple data mining approaches have been used in spectral data analysis, such as Principal Component Analysis (PCA), Random Forest (RF) and Support Vector Machine (SVM), all demonstrating the ability to discriminate diseased from non-diseased biofluid samples [44]. Currently, Partial Least Squares Regression analysis (PLSR) is one of the most frequently used techniques for the production of quantitative models, due to its ability to identify systematic variations of contributing factors and generate quantitative predictive models. This allows the prediction of unknowns, using the latent variables extracted from the regression model [40,32,45,46].

ATR-FTIR spectroscopy has the ability to detect minor differences in biofluid samples, with minimal sample preparation, and multiple proof-of-principle studies have highlighted the potential clinical use for such a technique. However, translation of ATR-FTIR spectroscopy has not occurred due to multiple factors, including the lack of acceptance from clinical environments.

We show, for the first time, an optimised methodology to enable protein quantification in single and complex mixtures using a PLSR approach, detailing the in-depth progression of determining protein concentration from spiked samples, to patient samples, before blind testing methods. The incorporation of this new quantification step within biofluid diagnostic methodologies would enable a direct comparison to gold standard diagnostic methods and highlight the clinical excellence of vibrational spectroscopic analysis of biofluids and facilitate translation.

2. Materials and methods

2.1. Sample preparation methodology

For the first time, an in depth methodological investigation was performed in order to establish the optimum sample preparation protocol for quantification from serum based ATR-FTIR spectroscopy. This study was performed using 2 models samples sets 1) Whole Serum Dilution Study and 2) Spiked Human Serum Models, before moving onto patient samples. Table 1 and subsections, 2.1.1 - 2.1.3, below provide further information on experimental details.