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Tuberculosis vaccine candidate: Characterization of H4-IC31 formulation and H4 antigen conformation

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ABSTRACT

Tuberculosis (TB) is one of the leading causes of death worldwide, making the development of effective TB vaccines a global priority. A TB vaccine consisting of a recombinant fusion protein, H4, combined with a novel synthetic cationic adjuvant, IC31®, is currently being developed. The H4 fusion protein consists of two immunogenic mycobacterial antigens, Ag85 B and TB10.4, and the IC31® adjuvant is a mixture of KLK, a leucine-rich peptide (KLKL5KLK), and the oligodeoxynucleotide ODN1a, a TLR9 ligand. However, efficient and robust methods for assessing these formulated components are lacking. Here, we developed and optimized phase analysis light scattering (PALS), electrical sensing zone (ESZ), and Raman, FTIR, and CD spectroscopy methods to characterize the H4-IC31 vaccine formulation. PALS-measured conductivity and zeta potential values could differentiate between the similarly sized particles of IC31® adjuvant and the H4-IC31 vaccine candidate and could thereby serve as a control during vaccine formulation. In addition, zeta potential is indicative of the adjuvant to antigen ratio which is the key in the immunomodulatory response of the vaccine. ESZ was used as an orthogonal method to measure IC31® and H4-IC31 particle sizes. Raman, FTIR, and CD spectroscopy revealed structural changes in H4 protein and IC31® adjuvant, inducing an increase in both the β-sheet and random coil content as a result of adsorption. Furthermore, nanoDSF showed changes in the tertiary structure of H4 protein as a result of adjuvantation to IC31®. Our findings demonstrate the applicability of biophysical methods to characterize vaccine components in the final H4-IC31 drug product without the requirement for desorption.

1. Introduction

Tuberculosis (TB) remains one of the leading causes of death worldwide with 10.4 million new cases reported in 2015. In the same year, 1.8 million people died from TB even with the availability of a vaccine [1]. The Bacillus Calmette-Guerin (BCG) vaccine is the only vaccine available against TB, despite its variable efficacy and its failure to control spread of the disease [1]. Although the BCG vaccine provides protection against manifestations of TB in children, it is not able to provide reliable protection against adult pulmonary TB [2]. Hence, there is an urgent need to develop a vaccine that can surpass the efficacy of the current BCG vaccine. One of the strategies in TB vaccine development is to modify or boost the immune response of the BCG vaccine [3–5]. Since BCG still confers protective immunity against TB in children, it is considered unethical to develop a vaccine strategy that does not include BCG [2].

One of the candidate TB vaccine formulations, H4-IC31 [6], is being developed by Sanofi Pasteur in collaboration with Statens Serum Institut (Copenhagen, Denmark) and Valneva (Lyon, France). The candidate vaccine's novel adjuvant IC31®, which acts as an immunostimulant, is composed of two biopolymers: an antimicrobial leucine-rich peptide (KLK or KLKL5KLK or KLKLLLLLKLK) and an oligodeoxynucleotide of repeating deoxyinosine and deoxycytosine dinucleotides (polyI:C) with a natural phosphodiester backbone (ODN1a) in a molar ratio of 25:1. The KLK peptide acts as a delivery vehicle by which it forms a delivery port for antigens at the injection site, making it a very interesting adjuvant concept [7]. KLK also facilitates the uptake and delivery of ODN1a into TLR9-positive intracellular vesicular compartments [8]. ODN1a co-localizes with

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TLR9 positive compartments, which are endosomes and endoplasmic reticular structures [8]. Due to these properties of IC31®, the adjuvant was shown to have a profound effect on immune response triggered by TLR9-agonists [9]. The combined effects of KLK and ODN1a stimulate antigen specific Th1 and/or Th17 adaptive memory through a TLR9 signaling pathway [10]. In this study, IC31® components were characterized using spectroscopic techniques.

The antigen protein component of the vaccine, H4, is a 41.3 kDa recombinant fusion protein consisting of Ag85 B and TB10.4 antigens from *Mycobacterium tuberculosis* [6,11]. The positive total surface charge of IC31® promotes the association with H4 protein through electrostatic interactions. The H4 protein and the individual components of IC31® adjuvant are water soluble. Once KLK and ODN1a are combined, micron-sized insoluble particles of IC31® are formed and remain insoluble upon formulation with H4. Consequently, the physical appearance of the IC31® adjuvant and H4-IC31 vaccine candidate are very similar. Due to the presence of these particles, many widely used analytical techniques that require soluble analytes cannot be applied. Moreover, biophysical methods that can be applied to samples containing particles may not be able to distinguish between IC31® adjuvant alone and H4-IC31 vaccine formulation.

Bioformulations such as vaccines are developed and assessed through a complex, multi-stage process. Key elements include: knowledge of underlying health and disease factors; knowledge of functional mechanisms of relevant bioformulations; development and production of candidate formulations; quality control involving product quality, safety, potency and stability; and delivery system for clinical trials and subsequent commercialization. One of the key steps in this process is to select and characterize both the individual components of these bioformulations as well as the final vaccine product. Characterization typically includes assessment of vaccine antigen structure throughout the manufacturing process. Stability assessment and quality control of antigen proteins require knowledge of antigen structure and of interactions with the surrounding matrix. Characterization of vaccine components at different manufacturing stages is critical as part of a quality control strategy [12].

Formulation of the vaccine product by adsorption of the antigen protein to the adjuvant is a critical manufacturing process step. Accordingly, a Phase Analysis Light Scattering (PALS) method was developed to characterize IC31® adjuvant and H4-IC31 vaccine formulation using zeta potential, conductivity, and particle size as reportable values. A particular advantage of this technology was its ability to distinguish between adjuvant alone and the adjuvanted vaccine. Protein conformational changes may result from physical adsorption to an adjuvant, and biophysical assays that are straightforward to apply to proteins in solution may be challenging in complex matrices such as adjuvants. Desorption of the antigen from the adjuvant prior to analysis may require buffer conditions which alter the protein structure and make subsequent interpretation of results problematic. In this study we report structural changes associated with H4 protein upon adsorption to IC31® adjuvant using CD, Raman, and FTIR spectroscopy, along with nano differential scanning fluorimetry (nanoDSF). The focus of this study was on the structural changes of the fully adsorbed H4 protein antigen, and not the adjuvant in excess, IC31®. Where the entire population of H4 undergoes structural changes as a result of adsorption, a majority of the IC31® population remains unaffected. Moreover, the constituents of IC31® function as immunostimulants, but do not exhibit any secondary structural elements on their own. IC31® is a much larger molecule (~3 μm) compared to H4 (12 nm) with a pseudo β-sheet structure, which is non-protein molecule containing elements that result in spectral features as β-sheet containing protein. Hence, any changes in pseudo β-sheet structure occurring in IC31® do not affect its function as an immunostimulant.

Raman spectroscopy was used to characterize the individual IC31® components, KLK and ODN1a. In summary, we have developed a panel of biophysical methods that can monitor the TB H4-IC31 vaccine adjuvantation process, and provide characterization information on formulated vaccine components without the need for desorption.

2. Materials and methods

All samples were obtained from different stages of the manufacturing process of the candidate vaccine product. The following samples were used without further modification unless otherwise stated: H4 antigen, IC31® adjuvant, and H4-IC31 [6,11]. Recombinant fusion H4 protein was purified as described in [13]. Its predicted pI value is 5.19, based on the amino acid sequence of individual AG85 B and TB10.4 proteins, as per the ExPASy portal. Adjuvant raw materials, KLK and ODN1a, were purchased from Bachem AG (Bubendorf, Switzerland) and Agilent Technologies Inc. (Boulder CO, USA) respectively. The buffer used for H4 and H4-IC31 was 10 mM Tris at pH 7.78. The molar ratio of H4:IC31 is 1:9, whereas IC31® consists of ODN1a:KLK in a 1:25 molar ratio. H4 was 1.2–1.4 mg/ml in purified samples, whereas after formulation H4 concentration decreases to 0.03 mg/ml. H4 is fully adsorbed (100%) to the IC31® adjuvant. A formulation containing higher concentration of H4 (0.1 mg/ml) had the same molar ratio of 1:9 and was fully adsorbed to the IC31® adjuvant. All measurements were performed in triplicate. More than three measurements were performed by PALS and ESZ.

2.1. Phase analysis light scattering (PALS)

Zeta potential is an essential parameter because depending on the magnitude of net charge, the stability of colloidal suspension can be assessed. When external potential is applied, the charged particle moves in the solution with altered velocity known as electrophoretic mobility. It depends on factors such as solvent viscosity and dielectric constant. The electrical potential at the boundary of diffuse layer is called zeta potential. The magnitude of this potential determines whether the repulsive forces exceed the attractive forces of the particles in colloidal solution, and this can ultimately be interpreted as stability of the colloidal solution.

An optical probe focuses a laser beam through the colloidal sample. The light scatters back to the probe from the particles. Zeta potential can be calculated using the Smoluchowski's equation.

$$\zeta = (\eta\mu)/\varepsilon$$

Where,
ζ: Zeta potential
η: solvent viscosity
μ: electrophoretic mobility
ε: dielectric constant of solvent

This method also measures the diameter of the particle based on the principles of dynamic light scattering (DLS), where the speed at which the particles are diffusing through solvent due to Brownian motion is measured by recording the fluctuations in the intensity of the scattered light. Using Stokes-Einstein equation one can estimate hydrodynamic radius or diameter. The particle size measurement is an intensity averaged distribution.

$$d(H) = kT/3\pi\eta D$$

where,
d(H): hydrodynamic radius of the particle
k: Boltzmann's constant

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