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Dipicolinic acid as a novel spore-inspired excipient for antibody formulation

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ABSTRACT

Ionic excipients are commonly used in aqueous therapeutic monoclonal antibody (mAb) formulations. Novel excipients are of industrial interest, with a recent focus on Arg salt forms and their application as viscosity reducing and stabilizing additives. Here, we report that the calcium salt of dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid), uniquely present in nature in the core of certain bacterial spores, reduces the viscosity of a mAb formulated at 150 mg/mL, below that achieved by Arg hydrochloride at the same concentration (10 mM). DPA also reduced the reversible phase separation of the same formulation, which characteristically occurs for this mAb upon cooling to 4 °C. Differential scanning calorimetry and differential scanning fluorimetry did not reveal a conformation destabilisation of the mAb in the presence of 10 mM DPA, or by the related quinolinic acid (QA, pyridine-2,3-dicarboxylic acid). However, fluorescence spectrophotometry did reveal localised (aromatic) conformational changes to the mAb attributed to DPA, dependent on the salt form. While precise mechanisms of action remain to be identified, our preliminary data suggest that these DPA salts are worthy of further investigation as novel ionic excipient for biologics formulation.

1. Introduction

Bacterial cells of the orders *Bacillales* and *Clostridiales* initiate the process of sporulation upon sensing conditions of nutrient limitation. Sporulation is a tightly regulated cellular differentiation process that results in the formation of an endospore (hereafter spore), a metabolically dormant cell-type that is adapted to resist physico-chemical and biological challenges for extended periods of time (Higgins and Dworkin, 2012; Tan and Ramamurthi, 2014). Whereas the primary protective feature of spores comprises an outermost proteinaceous coat that functions as a molecular barrier to chemical and enzymatic attack (McKenney et al., 2013), dormancy is achieved principally by two means. First, the cellular protoplast is surrounded by a thick layer of modified peptidoglycan, referred to as the cortex, which probably mechanically enforces the reduced water content of the spore core (Paredes-Sabja et al., 2011). Second, the spore core environment is highly mineralised with calcium and other divalent metal ions, which are chelated with pyridine-2,6-dicarboxylic acid (dipicolinic acid or DPA). The latter is uniquely associated with bacterial spores in nature, comprising approximately 10–15% of the dry weight of spores (Paredes-Sabja et al., 2011). While the precise function of DPA in spores has not definitively been elucidated, it clearly has a role in the maintenance of spore dormancy i.e. mutant strains of *Bacillus* that lack enzymes involved in DPA synthesis are unstable and lyse before maturity (Paredes-Sabja et al., 2011; Setlow, 2014). Similarly, the induced release of DPA from the spore core – whether achieved via nutrient induced germination, or by exposure of spores to certain cationic surfactants or extremely high pressures – results in the loss of spore dormancy and resistance properties (Setlow, 2014).

One potential role for DPA at the molecular level is to promote the stability of essential spore-core located proteins during dormancy and spore germination, perhaps by minimising thermal-induced motion and the likelihood of denaturation and aggregation. Given the apparent protective role of DPA in spores, we conjectured whether it would be able to fulfil a similar function as an excipient to pharmaceutical proteins of interest. The most commonly used pharmaceutical excipients include amino acids,

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polyols, salts, sugars, and surfactants (Manikwar et al., 2013). However, considerable effort has been made in recent years to identify and develop new excipients that mitigate the physical and chemical instability of biological drugs (Du and Klibanov, 2011; Lee et al., 2014). The use of novel Arg salts, for example, including equimolar formulations with glutamic acid, have been reported to exert synergistic effects in terms of reducing intermolecular attractions and aggregation, compared to Arg.HCl alone (Kheddo et al., 2016, 2014).

Here we report on the use of DPA, and its quinolinic acid (QA) analogue, as novel excipients that may have potential in mAb formulation. This class of biopharmaceutical is of particular interest since subcutaneous injections of mAbs comprise low volume (<1.5 mL) high protein concentration (>100 mg/mL) formulations. Such conditions promote aggregation, reversible self-association and particulate formation, with the resultant solubility and viscosity issues presenting considerable challenges to large-scale manufacture, product stability and delivery (Shire et al., 2004).

2. Materials and methods

2.1. Chemicals and reagents

All reagents were purchased at >98% purity. 2,3-Pyridinedicarboxylic acid (quinolinic acid or QA), 2,6-pyridinedicarboxylic acid (DPA), Bradford Reagent, calcium hydroxide, lysozyme and Scienceware® Aquet® liquid detergent were purchased from Sigma-Aldrich (Dorset, UK). Arg, Arg hydrochloride, histidine, histidine hydrochloride, and sodium chloride were acquired from J. T. Baker (Avantor Performance Materials B.V., Deventer, Netherlands). Calcium chloride dihydrate, SYPRO Orange protein gel stain 5000× in DMSO, and Tergazyme were obtained from Macron Chemicals (UK), Invitrogen (Paisley, UK) and Alconox (UK), respectively. MAb A is an IgG1 isotype with MW of 148.2 kDa, extinction coefficient or 1.443 cm^2/mg and pI 9.30. MAb B is an IgG1 isotype with MW of 148.4 kDa, extinction coefficient of 1.58 cm^2/mg and pI 7.5–7.8. MAb C is an IgG1 isotype with MW of 149.0 kDa, extinction coefficient of 1.55 cm^2/mg and pI 9.04. MAbs A, B and C were kindly provided by MedImmune Ltd., Cambridge, supplied at 50 mg/mL solution and stored at −80 °C until use.

2.2. Sample preparation

MAb A was defrosted on the bench and gently swirled to mix; 45 mL at 50 mg/mL was then dialysed against 5 L of 25 mM His, 120 mM NaCl, pH 6 buffer for two days using dialysis cassettes (Thermo Scientific, Slide-A-Lyzer, 30 kDa MWCO). Dialysis buffer was changed after 3 h and after 12 h dialysis. After dialysis, the protein concentration was determined by absorbance at 280 nm using a NanoDrop instrument (Thermo Fisher Scientific Inc., Wilmington, USA). mAb A was then concentrated to 150 mg/mL using a Microsep Advance Centrifugal Device with a 30 kD MWCO (Pall Corporation, NY, USA) in the absence or presence of different QA and DPA complexes. Before concentration, mAb A was diluted 1:2 using (1) 20 mM $Ca(OH)_2$ 20 mM QA; (2) 20 mM $Ca(OH)_2$ 20 mM DPA; (3) 20 mM $CaCl_2{\cdot}2H_2O$; (4) 40 mM Arg 20 mM QA; (5) 40 mM Arg 20 mM DPA; (6) 40 mM Arg.HCl. Since both QA and DPA absorb ultra-violet light, the protein concentration was determined by Bradford assay. Briefly, protein samples were diluted 2000× in the same buffer they were prepared in and 50 μL of each sample added to a 96-well microplate. A calibration curve (0–0.25 mg/mL) was prepared using mAb A in 25 mM His, 120 mM NaCl, pH6. 200 μL of Bradford reagent was added to each well before incubating with mild agitation at room temperature in the dark for 20 min. Sample absorbance was read subsequently at 595 nm.

2.3. Determination of mAb A thermal stability by differential scanning calorimetry (DSC)

1 mg/mL mAb A samples were prepared in buffers containing various QA and DPA complexes at a range of concentrations. The following stock solutions used to prepare the samples: 50 mg/mL mAb A in 25 mM His, 120 mM NaCl, pH 6; 20 mM DPA (or QA) in 90 mM His, 120 mM NaCl, pH 6; 20 mM $Ca(OH)_2$, 20 mM DPA (or QA), 25 mM His, 120 mM NaCl, pH6; 40 mM Arg, 20 mM DPA (or QA), 25 mM His, 120 mM NaCl, pH6. Buffer strength had to be increased to 90 mM His instead of 25 mM His in order to maintain a stable pH in the presence of 20 mM DPA or QA free acids. Samples were prepared using these stock solutions and then diluted in 25 mM His, 120 mM NaCl, pH 6 to achieve the desired QA and DPA concentrations. Buffers and MilliQ water were degassed by sonication, whereas protein samples were degassed using a degassing station (TA Instruments). Lysozyme was used as a reference protein. 900 μL of each sample and buffer were pipetted into a 96-deepwell plate and samples were heated from 25 to 90 °C using a rate of 2 °C/min and an equilibration time of 600 s using a NanoDSC differential scanning calorimeter (TA Instruments). Data analysis was performed in NanoAnalyze 3.6.0 software (TA Instruments). DSC curves were fitted using a two-state model of three independent domains to determine the melting temperature (T_m (°C)) and enthalpy difference (ΔH (KJ/mol) during thermal denaturation of each domain. The entropy difference (ΔS (KJ/mol. K)) was determined using the following equation: $\Delta S = \Delta H/(T_m + 273.15)$

2.4. Determination of tertiary structure modifications by intrinsic fluorescence spectroscopy

1 mg/mL mAb A in DPA (or QA), Ca^{2+}-DPA (or QA), Arg-DPA (or QA) (2:1) with DPA or QA concentrations ranging from 0 to 10 mM were prepared before adding 300 μL of each sample to a black 96-well microplate (in sextuplicates). Fluorescence emission was monitored between 300 and 400 nm using a Hitachi F-7000 fluorescence spectrophotometer. Stock solutions and samples were prepared as in **2.3**.

2.5. Assessing the conformational stability of mAb A solutions by differential scanning fluorimetry (DSF)

An intermediate protein stock solution was prepared by adding 2 μL SYPRO Orange (5000×) to 49 μL of 50 mg/mL mAb A and 144 μL 25 mM His, 120 mM NaCl, pH 6 buffer to a final protein concentration of 12.5 mg/mL. The sample was vortexed and protected from light. The intermediate protein stock solution (2 μL) was pipetted to individual wells of a 96-well microplate along with 23 μL of each buffer of interest (in triplicates). The buffers consisted of 25 mM His, 120 mM NaCl, pH 6 with: 1 mM, 5 mM and 10 mM of DPA or QA; 10 mM $Ca(OH)_2$, 10 mM DPA; 10 mM $Ca(OH)_2$, 10 mM QA; 10 mM $CaCl_2{\cdot}2H_2O$; 20 mM Arg.HCl; 20 mM Arg, 10 mM DPA; 20 mM Arg, 20 mM QA. Thermal unfolding of mAb A then monitored using a Biorad CFX96 Real-Time PCR system using a temperature range of 20 °C to 95 °C with an increment of 0.2 °C.min^{-1} and a hold time of 10 s. As mAb A unfolds, SYPRO Orange binds to its exposed hydrophobic surfaces, resulting in an increase of fluorescence. Two unfolding events most likely associated with unfolding of CH2 and CH3 domains of mAb A are observed at temperature of hydrophobicity 1 (Th1) and temperature of hydrophobicity 2 (Th2), respectively.