International Journal of Pharmaceutics 473 (2014) 126–133

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

The effect of arginine glutamate on the stability of monoclonal antibodies in solution

Priscilla Kheddo [a,b], Malgorzata Tracka [c], Jonathan Armer [c], Rebecca J. Dearman [b], Shahid Uddin [c], Christopher F. van der Walle [c], Alexander P. Golovanov [a,b,*]

[a] Manchester Institute of Biotechnology, University of Manchester, Manchester M1 7DN, UK
[b] Faculty of Life Sciences, University of Manchester, Manchester M13 9PL, UK
[c] MedImmune Ltd., Granta Park, Cambridge CB21 6GH, UK

ARTICLE INFO

Article history:
Received 21 May 2014
Received in revised form 26 June 2014
Accepted 27 June 2014
Available online 30 June 2014

Keywords:
Protein aggregation
Excipients
mAbs formulation
Physical characterization
Physical stability

ABSTRACT

Finding excipients which mitigate protein self-association and aggregation is an important task during formulation. Here, the effect of an equimolar mixture of L-Arg and L-Glu (Arg·Glu) on colloidal and conformational stability of four monoclonal antibodies (mAb1–mAb4) at different pH is explored, with the temperatures of the on-set of aggregation (T_{agg}) and unfolding (T_{m1}) measured by static light scattering and intrinsic fluorescence, respectively. Arg·Glu increased the T_{agg} of all four mAbs in concentration-dependent manner, especially as pH increased to neutral. Arg·Glu also increased T_{m1} of the least thermally stable mAb3, but without similar direct effect on the T_{m1} of other mAbs. Raising pH itself from 5 to 7 increased T_{m1} for all four mAbs. Selected mAb formulations were assessed under accelerated stability conditions for the monomer fraction remaining in solution after storage. The aggregation of mAb3 was suppressed to a greater extent by Arg·Glu than by Arg·HCl. Furthermore, Arg·Glu suppressed the aggregation of mAb1 at neutral pH such that the fraction monomer was near to that at the more typical formulation pH of 5.5. We conclude that Arg·Glu can suppress mAb aggregation with increasing temperature/pH and, importantly, under accelerated stability conditions at weakly acidic to neutral pH.

1. Introduction

Monoclonal antibodies (mAbs) are promising therapeutic drugs for the treatment of a wide range of diseases (Chan and Carter, 2010; Leavy, 2010; Smith and Clatworthy, 2010; Weiner et al., 2010). Their success is due to various properties including their high binding specificity and affinity, robust manufacturing processes, and the availability of humanized forms that attenuate immunogenic responses (Beck et al., 2010). However, mAb self-association and aggregation is sometimes observed during formulation at high concentrations (>100 mg/ml), and also with environmental stresses such as shaking, changes in solution pH, freeze–thaw and elevated temperatures (Banga, 2006; Chaudhuri et al., 2014; Cromwell et al., 2006; Wu et al., 2014). Hence, optimization of a mAb formulation by controlling buffer, ionic strength and pH as well as the addition of excipients is crucial in minimizing the extent of aggregation (Aboel Dahab and El-Hag, 2014; Goldberg et al., 2011; He et al., 2011; Saito et al., 2013; Sule et al., 2012).

All globular proteins, including mAbs are known to be susceptible to aggregate formation. The term 'protein aggregation' can be defined in a broad sense as any pathway forming protein assemblies, or aggregates (Mahler et al., 2009). Aggregation may result from the reversible self-association of the native protein, or irreversible formation of non-native assemblies following the partial or complete unfolding. (Aggregation through changes in post-translational modification and chemical degradation will not be considered here.) Self-association involving native protein–protein interaction may occur through complementary surface effects, attractive electrostatic or short range attractive forces (He et al., 2011; Liu et al., 2005). Such intermolecular self-association of proteins is related to the colloidal stability, which can be assessed by, for example, measuring temperature at which light becomes scattered by protein aggregates appearing. Aggregation involving partially unfolded protein may occur via exposed hydrophobic patches, generating non-native assemblies, and is related to the conformational stability of a protein (Arzenšek et al., 2012; Goldberg et al., 2011; Pace et al., 1996; Shi et al., 2013).

* Corresponding author at: Manchester Institute of Biotechnology and Faculty of Life Sciences, The University of Manchester, 131 Princess Street, Manchester M1 7DN, UK. Tel.: +44 161 306 5813; fax: +44 161 306 5201.
E-mail address: A.Golovanov@manchester.ac.uk (A.P. Golovanov).

http://dx.doi.org/10.1016/j.ijpharm.2014.06.053
0378-5173/

Conformational stability can be assessed by measuring the temperature of protein melting transition. Ideally, increasing both the colloidal and conformational stability would be beneficial for creating a stable formulation, however in practice optimizing one of these parameters may compromise the other.

When considering approaches to choosing mAbs formulation for the best stability, the correct selection of buffer, pH and excipient(s) is essential. The solution pH can have profound effects on protein structure, stability and biological activity (Kopec and Schneider, 2011; Thakkar et al., 2012). In the context of formulation, pH is optimized to minimize physical and chemical degradation pathways (Cromwell et al., 2006; Gokarn et al., 2008). Generally, mAbs with a pI around 8–9 are formulated in mildly acidic buffer, avoiding for example deamidation and aggregation sometimes occurring in mildly alkaline buffer. These conditions however are not necessarily the best for the optimal conformational stability. Another difficulty arises from the limited choice of excipients available for formulation of pharmaceutical mAbs: only those listed as Generally Recognized as Safe (GRAS) by the regulatory bodies are used in practice (Ogaji et al., 2011; Pifferi and Restani, 2003). Presently, the process of formulation (i.e. choosing the best solution conditions and excipients) takes into account the protein's physiochemical properties and may also involve high-throughput screening (Li et al., 2011). Although there is no 'universal excipient' able to stabilize all the proteins, discovering a combination of excipients which would be applicable for a wider range of proteins is highly desirable.

An equimolar combination of the free amino acids L-arginine and L-glutamic acid (Arg·Glu) has been previously suggested (Golovanov et al., 2004) as a way to increase the solubility limit and long-term stability of several diverse proteins prone to aggregation; since then the method has been widely adopted in protein structural and functional studies (Blobel et al., 2007, 2011; Hautbergue and Golovanov, 2008; Valente et al., 2005; Vedadi et al., 2006). L-Arginine itself (normally used in a form of a hydrochloride salt, Arg·HCl, to bring its solution pH down to neutral) is a widely known additive which often is used to assist protein refolding and reduce aggregation and solution viscosity (Arakawa et al., 2007; Chen et al., 2008; Das et al., 2007; Fukuda et al., 2014; Liu et al., 2005; Schneider et al., 2011; Vagenende et al., 2013). However a number of studies established that on a per-mole basis, Arg·Glu is much more effective at reducing intermolecular attractions and aggregation than L-Arg (Golovanov et al., 2004; Valente et al., 2005; Vedadi et al., 2006). The mechanism of Arg·Glu effect has been investigated using experimental (Blobel et al., 2011) and in silico methods (Shukla and Trout, 2011), which explained the synergy of the action of L-Arg combination with L-Glu. The significant effect of Arg·Glu on preventing protein aggregation is observed already at 50 mM (Golovanov et al., 2004), with an in silico study suggesting that an "optimum" concentration for an anti-aggregation effect may exist in the range of 100–200 mM, at least for the protein used for the simulations (Shukla and Trout, 2011). Recently, the stabilizing effect of high concentrations (up to 0.5 M) of Arg·Glu versus Arg·HCl on a selected IgG1 has been explored which suggested that having L-Glu (or L-Asp) as counterions counteracts the potentially disadvantageous destabilizing effects of L-Arg (Fukuda et al., 2014). Despite the growing popularity of using Arg·Glu as excipients for increasing protein solubility and preventing protein aggregation, to our knowledge, the systematic studies of their utility for diverse mAbs in the context of formulation as pharmaceuticals has not yet been reported.

Here we used high-throughput analysis to screen the aggregation propensity and thermal stability of mAbs in a variety of conditions. We first investigated the concentration- and pH-dependent effect of Arg·Glu (in the pharmaceutically-acceptable osmolality range) on the temperatures of the on-set of aggregation (T_{agg}) and first melting transition (T_{m1}) of four IgG1 mAbs as assessed by static light scattering (SLS) and intrinsic fluorescence, respectively. The effect of buffer type and solution pH on the stability of selected mAb formulations was then explored under accelerated stability conditions (storage at elevated temperature for a number of weeks), analysed for the fraction monomer by size exclusion high pressure liquid chromatography (SE-HPLC). The results suggest that using Arg·Glu as excipient at concentrations <200 mM can reduce temperature-induced aggregation of mAbs especially at pH approaching neutral, where the inherent conformational stability of mAbs is theoretically higher.

2. Materials and methods

2.1. Monoclonal antibodies and sample preparation

The four different mAbs (IgG1 with MWs from ~145 to 148 kDa) tested here were kindly provided by MedImmune. The isoelectric points (pI) of mAb1, mAb2, mAb3 and mAb4 are 7.9–8.3, 8.44, 8.56 and 8.53, respectively; all values were measured experimentally except for that of mAb2 which was calculated. For SLS and intrinsic fluorescence measurements, the mAbs were diluted to 1 mg/mL in 10 mM citrate–phosphate (C–P) buffer (pH 5–7). These solutions were supplemented with varying concentrations of Arg·Glu (50–200 mM) as required, using prepared 1 M stock solution (Golovanov et al., 2004) containing equimolar mixture of the free amino acids L-Arg (Analytical grade, Sigma–Aldrich) and L-Glu (USP-FCC grade, J.T. Baker) in MilliQ water (18.2 MΩ cm), with pH adjusted where necessary. For preparation of buffers containing Arg·HCl, the hydrochloride salt of L-Arg was used (USP-FCC grade, J.T. Baker). The mAbs were diluted to 0.5 mg/mL for absorption measurements at 280 nm. For SE-HPLC, mAbs were diluted to 10 mg/mL in the appropriate buffer.

2.2. Determining solution osmolality

The osmolality of Arg·Glu solutions in the presence and absence of a mAb2 was measured using an Osmomat 030-D Cryoscopic Osmometer (Gonotec GmbH, Berlin, Germany). Measurement results are shown in Supplementary Information, Fig. S1. Arg·Glu concentrations of 5, 10, 25, 50, 100, 150 and 200 mM were prepared from a 1 M stock in MilliQ water, and also at concentrations of 50, 150 and 200 mM in 10 mM C–P buffer, pH 6.0. For solutions containing protein, mAb2 was buffer exchanged using overnight dialysis into 10 mM C–P buffer, pH 6.0, containing Arg·Glu concentrations of 50, 150 and 200 mM. The protein concentration was adjusted by dilution with the appropriate Arg·Glu solution to 30 mg/ml; concentrations were verified in triplicate using a NanoDrop 2000 (Thermoscientific, Stafford House, Hertfordshire), by measuring optical absorption at 280 nm.

2.3. Static light scattering and intrinsic fluorescence

SLS and intrinsic fluorescence measurements were conducted simultaneously using an Optim 2 (Avacta, Thorp Arch Estate, Wetherby). Data was processed using the standard Optim analysis software provided (Avacta, 2013a), as per manufacturer's recommendations (Avacta, 2013b). Briefly, the SLS at 266 nm was used as an indicator for "colloidal stability", reporting the onset of aggregation temperature (T_{agg}), which can be defined as the temperature at which the measured scatter reaches a threshold that is approximately 10% of its maximum value (for typical trace, see Supplementary Information, Fig. S2). The changes in the SLS signal represented changes in the weight average molecular mass observed due to protein aggregation. The conformational stability