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Antimicrobial preservatives induce aggregation of interferon alpha-2a: The order in which preservatives induce protein aggregation is independent of the protein

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Abstract

Antimicrobial preservatives (APs) are included in liquid multi-dose protein formulations to combat the growth of microbes and bacteria. These compounds have been shown to cause protein aggregation, which leads to serious immunogenic and toxic side-effects in patients. Our earlier work on a model protein cytochrome *c* (Cyt c) demonstrated that APs cause protein aggregation in a specific manner. The aim of this study is to validate the conclusions obtained from our model protein studies on a pharmaceutical protein. Interferon α-2a (IFNA2) is available as a therapeutic treatment for numerous immune-compromised disorders including leukemia and hepatitis c, and APs have been used in its multi-dose formulation. Similar to Cyt c, APs induced IFNA2 aggregation, demonstrated by the loss of soluble monomer and increase in solution turbidity. The extent of IFNA2 aggregation increased with the increase in AP concentration. IFNA2 aggregation also depended on the nature of AP, and followed the order m-cresol > phenol > benzyl alcohol > phenoxyethanol. This specific order exactly matched with that observed for the model protein Cyt c. These and previously published results on antibodies and other recombinant proteins suggest that the general mechanism by which APs induce protein aggregation may be independent of the protein.

Keywords

Interferon alpha-2a; preservatives; aggregation; formulation; benzyl alcohol; m-cresol

1. Introduction

Multi-dose protein formulations comprise approximately one third of protein-based pharmaceuticals available on the global market (Meyer et al. 2007). These formulations are

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beneficial in terms of economics and patient compliance, and require the inclusion of at least one antimicrobial preservative (AP) in order to inhibit the growth of microbes and bacteria during administration (Akers et al. 2002, Meyer et al. 2007).

It has become increasingly important to study APs in protein pharmaceuticals because these necessary compounds have been linked to protein aggregation in the liquid state. One of the earliest reports demonstrated that the addition of various aromatic compounds induced the aggregation of recombinant human growth hormone (Maa and Hsu 1996). Numerous studies have shown the ability of APs to cause destabilization and aggregation of many proteins (Gupta and Kaisheva 2003, Tobler et al. 2004, Zhang et al. 2004, Roy et al. 2005). Aggregates in these formulations cause a decrease in the effective concentration of the delivered drug as well as result in immunogenic and toxic responses in patients (Ratner et al. 1990, Bucciantini et al. 2002, Hermeling et al. 2006, Rosenberg 2006, Fradkin et al. 2009, Sauerborn et al. 2010, Vazquez-Rey and Lang 2011). In order to minimize AP-induced protein aggregation, an understanding of the interactions between APs and proteins is critical.

Previous work from our laboratory has demonstrated that a specific order exists in which individual APs induce the aggregation of a model protein cytochrome *c* (Cyt c) (Hutchings et al. 2013). The aim of the present study is to examine the effects of various APs on a pharmaceutically relevant protein and validate the results obtained with the model protein. We chose interferon α-2a (IFNA2) (Figure 1) for this purpose, which is available as a therapeutic treatment for numerous immune-compromised disorders including leukemia and hepatitis C. IFNA2 has been shown to aggregate in its formulation state (Braun and Alsenz 1997, Braun et al. 1997, Hochuli 1997, Ryff 1997). APs have been used in IFNA2 multi-dose as well as single-dose liquid formulations. However, it is unknown whether APs cause IFNA2 aggregation. Here, we demonstrate that IFNA2 aggregation is enhanced in the presence of APs and that the extent and order of these effects match exactly to what we observed earlier in the case of the model protein Cyt c (Hutchings et al. 2013).

2. Materials and Methods

2.1 Materials

Synthetic cDNA corresponding to IFNA2 was obtained from Operon (Huntsville, Alabama), and was cloned into the pET-SUMO expression vector (a generous gift from Christopher Lima, Sloan-Kettering Institute). Protein was expressed in *Escherichia coli* BL21(DE3) cells, and the soluble protein was purified using a Nickel Sepharose 6 Fast Flow column (GE Healthcare Life Sciences, Pittsburgh, Pennsylvania). The SUMO tag was cleaved using the Ulp1 protease, leaving no additional amino acids. The final protein sequence is identical to that of the pharmaceutical protein. Detailed expression and purification protocols, and biophysical characterization of the protein were described in our earlier publication (Bis et al. 2014).

Preservatives were obtained in their highest available purity (Table 1). All experiments were performed in the buffer conditions used for IFNA2 formulations (0.01 M ammonium acetate, 0.12 M sodium chloride, pH 5.0), unless otherwise noted.

2.2 Preservative Efficacy Test

To confirm the antimicrobial activity of APs, a simplified preservative efficacy test was performed (Sutton and Porter 2002, Hutchings et al. 2013). A primary culture of *Escherichia coli* BL21(DE3) cells was incubated overnight at 37°C in a shaker. Aliquots of 0.5 ml were transferred into five 50 ml culture flasks containing either no preservative (control), or one of the four APs (Table 1). Cultures were incubated at 37°C with shaking for six hours, and optical density at 600 nm was used to measure the cell count.

2.3 Size Exclusion Chromatography

To monitor the effects of various APs on protein aggregation, IFNA2 (10 μM in 0.01 M ammonium acetate, 0.12 M sodium chloride, pH 5) was incubated at 50°C in borosilicate vials (Kimble Chase Life Science, #60910 L-12, Vineland, New Jersey) and samples were taken at desired intervals. Samples were centrifuged to remove insoluble aggregates prior to HPLC injection. Concentration of monomer was estimated by injecting 70 μL onto a TSKgel 5 μM G3000SWxl column (Tosoh Bioscience LLC, San Francisco, California) on an Agilent 1100 HPLC (Santa Clara, California) at room temperature. The mobile phase used was 0.01 M ammonium acetate, 0.12 M sodium chloride, pH 5, at a flow rate of 1 mL min^{-1}. Absorbance at 280 nm was used to determine the monomer content.

2.4 Isothermal Incubation Experiments

IFNA2 (10 μM) was incubated at the desired temperature with various APs, and the changes in optical density at 350 nm were measured as a function of the incubation time (Eberlein et al. 1994, Eckhardt et al. 1994). Buffer and protein do not absorb at this wavelength. The aggregation kinetics were monitored until the signal reached a plateau. At longer incubation times, the aggregates started to settle down to the bottom of the cuvette, resulting in decreased optical density. At that point, the experiment was stopped.

2.5 Thermal Scanning Method

The aggregation temperature (T_m^{Agg}) of the protein was measured on an UV-Visible spectrophotometer (Agilent Technologies, Santa Clara, California). The temperature was increased at a rate of 1°C/min followed by 90 sec equilibration, and changes in the optical density at 450 nm were recorded (Charman et al. 1993, Kurganov 2002). T_m^{Agg} was determined as the temperature at half the maximum optical density. For these experiments, 10 μm IFNA2 in formulation buffer was used with varying AP concentration.

3. Results

3.1 APs Cause IFNA2 Aggregation

Four commonly employed APs in liquid protein formulations were used in this study. Table 1 lists the concentrations of these APs used in formulations. Figure 2A shows the antimicrobial efficacy of these APs, measured using a simplified test (Sutton and Porter 2002, Hutchings et al. 2013). We tested their effect on the growth of *Escherichia coli* bacteria. For this purpose, we used BL21(DE3) cells in LB media and monitored the cell count by measuring changes in the optical density at 600 nm as a function of the growth

time. Without APs, the growth curve showed an exponential increase (Figure 2A). However, the optical density increase was minimal with the addition of any of the four APs when compared with the growth curve with no AP, indicating that these molecules inhibited bacterial growth. Table 2 lists the minimum inhibitory concentration (MIC) values for these APs against various organisms (Lucchini et al. 1990, Simpson and Wuthiekanun 2000, Meyer et al. 2007, Rowe et al. 2009, Abd-Elsalam et al. 2011).

In previous studies, APs have been shown to induce protein aggregation over a period of days or months (Maa and Hsu 1996, Katakam and Banga 1997, Remmele Jr. et al. 1998, Gupta and Kaisheva 2003, Tobler et al. 2004, Roy et al. 2006, Thirumangalathu et al. 2006). In order to test the effects of different APs on protein aggregation on a convenient laboratory timescale, we accelerated aggregation kinetics by conducting isothermal incubation studies at an elevated temperature. Using elevated temperatures to accelerate protein aggregation is becoming a commonly used method for scanning the effect of a large number of solution conditions on protein stability and aggregation (Brummitt et al. 2011, Nashine et al. 2013, Chaudhuri et al. 2014). Initially, we demonstrated that APs induce IFNA2 aggregation. For comparing the effect of the four APs on IFNA2 aggregation, we chose a concentration of 0.3% v/v AP due to the low solubility of m-cresol above this concentration. At this concentration of 0.3% v/v, all the APs retained their antimicrobial efficacy (Figure 2B). Isothermal aggregation kinetics were performed at 50°C and the concentration of IFNA2 monomer was monitored as a function of the incubation time (Figure 3). With all the APs, only soluble monomer was detected using SE-HPLC. After a period of one day in the absence of AP, approximately 90% IFNA2 monomer remained in solution. No monomer was detected after eight hours in the presence of 0.3% v/v m-cresol (CR) and after 12 hours in the presence of 0.3% v/v phenol (PH). Both benzyl alcohol (BA) and phenoxyethanol (PE) exerted a marginal effect on monomer loss, losing approximately 20% monomer content over the course of 24 hours. To differentiate the impact of BA and PE on IFNA2 monomer loss, an isothermal incubation study was performed using 0.9% v/v AP concentration (Figure 3 inset). In 0.9% BA, all IFNA2 monomer disappeared within eight hours at 50°C, while monomer was detected up to eight hours in PE samples. These results followed the order CR > PH > BA > PE in terms of their effects on IFNA2 aggregation. We also monitored the change in optical density as a function of the incubation time, in order to correlate monomer loss with protein aggregation (Figure 4). In these cases, the same pattern of AP-induced destabilization was observed: CR > PH > BA > PE. No IFNA2 aggregation was observed in the absence of APs. This order in which APs induce IFNA2 aggregation is identical to what we observed earlier in the case of the model protein Cyt c: CR > PH > BA > PE (Hutchings et al. 2013).

3.2 IFNA2 Aggregation Increases with AP Concentration

Another method used in the literature to monitor the effect of solution conditions on protein aggregation is thermal scanning (Brummitt et al. 2011, Nashine et al. 2013, Chaudhuri et al. 2014). This is very similar to using denaturant melts to determine the protein stability, although denaturant is not present in protein formulations. Here, we used temperature as the perturbant, rather than the denaturant. We utilized the thermal scanning technique to determine the effects of preservative concentration on IFNA2 aggregation. We measured the

changes in the optical density at 450 nm as a function of increasing solution temperature. This wavelength was selected because neither the protein nor any solution components absorb in this range, and the changes in optical density can be attributed solely to protein aggregation. In buffer alone without APs, the optical density initially increased with temperature in a sigmoidal variation (Figure 5). At higher temperatures, aggregated protein particles began to settle to the bottom of the cuvette, causing an observable decrease in the optical density. We performed these thermal scanning experiments in the presence of increasing concentrations of PH (Figure 5). With the inclusion of PH, the midpoint aggregation temperature (T_m^{Agg}) decreased. In the absence of PH, the T_m^{Agg} of IFNA2 was 63.9 ± 0.9°C. The addition of 0.5% PH decreased the T_m^{Agg} to 56.7 ± 0.7°C, indicating that the presence of PH accelerated the aggregation of IFNA2. A similar phenomenon was observed with Cyt c whose aggregation increased with an increase in AP concentration (Hutchings et al. 2013).

3.3 Aggregation Temperature Decreases Linearly with AP Concentration

As seen with PH, the other APs also caused a decrease in T_m^{Agg} of IFNA2 with the increase in AP concentration (Figure 6). Interestingly, a linear correlation was observed between the T_m^{Agg} and the concentration of AP. This relationship is significant in that it is a qualitative measurement of how efficiently each AP causes IFN aggregation, which is represented in their individual slopes. This slope is analogous to the m-value commonly used in analyzing protein denaturant melts (Santoro and Bolen 1988), which represents the efficiency of a denaturant to denature a protein. Comparison of the slopes of T_m^{Agg} variation with the AP concentration demonstrates an effective order of CR > PH > BA > PE, with CR being the most effective AP in causing protein aggregation. These experiments indicate that for every percent concentration change of CR, the T_m^{Agg} of IFNA2 decreases by 22°C. In contrast, PE has a slope of 8.2°C/%v/v, nearly three-fold less in its effectiveness to aggregate IFNA2 than CR. This trend matches to that observed in the isothermal incubation studies (Figures 3 & 4), and is identical to our earlier observations on the model protein Cyt c (Hutchings et al. 2013).

4. Discussion

Protein therapeutics are marketed in a variety of dosage forms, including multi-dose formulations, which require the inclusion of at least one antimicrobial preservative in order to combat the growth of microbes and bacteria during repeated contact between the solution and a syringe needle (Cleland et al. 1993, Akers et al. 2002, Meyer et al. 2007). However, it has been shown that these preservatives cause protein aggregation (Maa and Hsu 1996, Katakam and Banga 1997, Remmele Jr. et al. 1998, Gupta and Kaisheva 2003, Tobler et al. 2004, Roy et al. 2006, Thirumangalathu et al. 2006). We have demonstrated previously that APs used in liquid protein formulations lead to protein destabilization and aggregation using the model protein Cyt c (Singh et al. 2010, Singh et al. 2011, Hutchings et al. 2013). The extent of this effect was dependent upon the nature of the AP, and the pattern of aggregation observed was CR > PH > BA > PE.

In this study, we tested the conclusions drawn from our model protein studies on a pharmaceutically relevant protein interferon α-2a. IFNA2 belongs to a family of cytokines

that play crucial roles in the innate immune response, and is one of the numerous interferon-α subtypes found in humans. A number of interferon-α products exist in the pharmaceutical market and are used to treat various debilitating diseases including hairy cell leukemia and hepatitis c (Hiscott et al. 1984, Diaz et al. 1993, Kirkwood 2002).

IFNA2 multi-dose formulations have been shown to aggregate in the liquid state (Braun and Alsenz 1997, Braun et al. 1997, Hochuli 1997, Ryff 1997) and contain APs. In order to validate the conclusions drawn from our studies on a model protein Cyt c, we studied the effect of multiple APs on IFNA2 aggregation using various biophysical techniques. Isothermal incubation studies showed that APs cause the loss of IFNA2 monomer (Figure 3), and the increase in optical density due to protein aggregation (Figure 4). The order in which APs induce IFNA2 aggregation was CR > PH > BA > PE, which is the same order we observed earlier in the case of the model protein Cyt c (Hutchings et al. 2013). Further, the addition of AP caused a decrease in T_m^{Agg}. The slopes indicate that CR was the most efficient in aggregating IFNA2, whereas PE was the least (Figure 6). Again, this order exactly matched the pattern we observed in our earlier studies on the model protein Cyt c (Hutchings et al. 2013). Based on these results, PE appears to be the best preservative choice for pharmaceutical formulations in causing less protein aggregation when compared with the commonly used BA. However, the perfect AP and its concentration in a multi-dose formulation should be such that it causes less protein aggregation and has sufficient antimicrobial activity (Hutchings et al. 2013).

Similar comparisons, although not as extensive as demonstrated in the case of IFNA2 or Cyt c, were made earlier on other proteins. Recombinant human growth hormone (rhGH) aggregation was monitored during freezing, high-temperature incubation, and agitation using changes in optical density and the percentage monomer loss by SEC. In this case, the three APs followed a similar order in causing rhGH aggregation: CR >PH >BA (Maa and Hsu 1996). In the case of a monoclonal antibody (IgG) (Gupta and Kaisheva 2003), aggregation was monitored during isothermal incubation using visual inspection of the samples, percent monomer loss using SEC, and light scattering. The three APs examined induced protein aggregation in the order: CR> PH> BA. Changes in interleukin-1 receptor (IL-1R) melting temperature assessed by DSC, and monomer content measured using SEC in the presence of preservatives also showed a similar relationship (Remmele Jr. et al. 1998). Taken together, the data on these five proteins (Cyt c, IFNA2, rhGH, IgG, IL-1R), suggest that the order in which APs induce protein aggregation may be independent of the nature of protein. Cyt c, IFNA2, and rhGH are α-helical proteins, whereas the IgG is primarily β-sheet and IL-1R contains both α-helix and β-sheet. The proteins also differ in their function: Cyt c plays a role in electron transport; IFNA2, IgG, and IL-1R have critical functions in the immune system; and rhGH is a multi-functional protein with emphasis on growth and regeneration.

The observation that the extent of AP effects on protein aggregation is independent of the nature of protein implies that APs may interact with common structural groups present in proteins. APs do not have strong binding sites on proteins (Maa and Hsu 1996, Roy et al. 2006, Singh et al. 2010, Singh et al. 2011). APs may hydrogen bond with the peptide backbone, suggested by earlier studies on BA and polyproline monitoring changes in amide

I, amide II, and hydroxyl bands using infrared spectroscopy (Strassmair et al. 1969). The extent of aggregation seems to qualitatively correlate with the hydrophobicity of the AP (Singh et al. 2011, Hutchings et al. 2013). APs also have been suggested to interact with hydrophilic regions of proteins (Alford et al. 2011). Perhaps the mechanism by which APs affect protein aggregation is a combination of hydrogen bonding, hydrophobic interactions, and electrostatics, and further examination of the exact mechanism is necessary in order to develop stable and effective pharmaceuticals.

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Abbreviations

BA	benzyl alcohol
Cyt c	cytochrome c
CR	m-cresol
IFNA2	interferon alpha-2a
PE	phenoxyethanol
PH	phenol
SE-HPLC	size exclusion high pressure liquid chromatography

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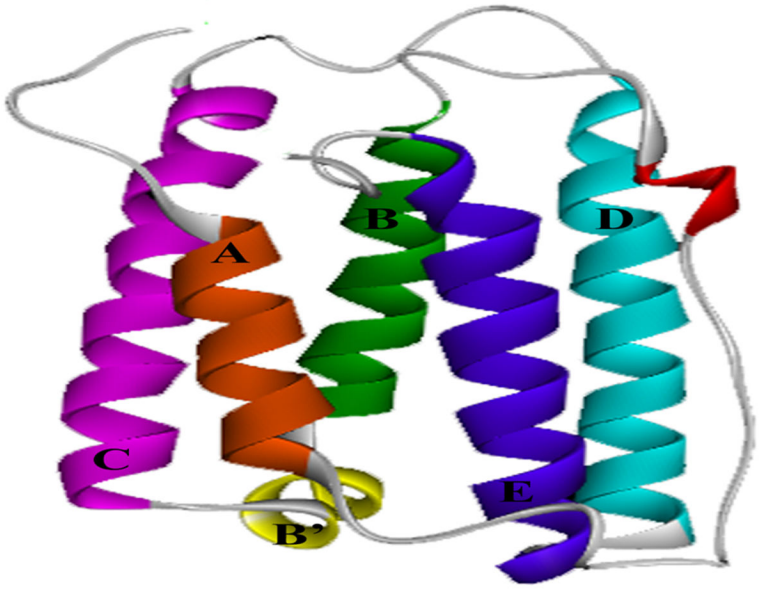


Figure 1.

Molecular structure of interferon α-2a (IFNA2; 1ITF.pdb). The protein is α-helical in nature. In the structure, helices are colored according to their organization in the three-dimensional structure: helix A, residues 11–21 (orange); helix B, residues 52–68 (green); helix B′, residues 70–75 (yellow); helix C, residues 78–100 (purple); helix D, residues 110–132 (cyan); helix E, residues 137–157 (blue). Residues 22–51 comprise the AB-loop, with residues 40–43 usually found in a 3_{10} helix (red).

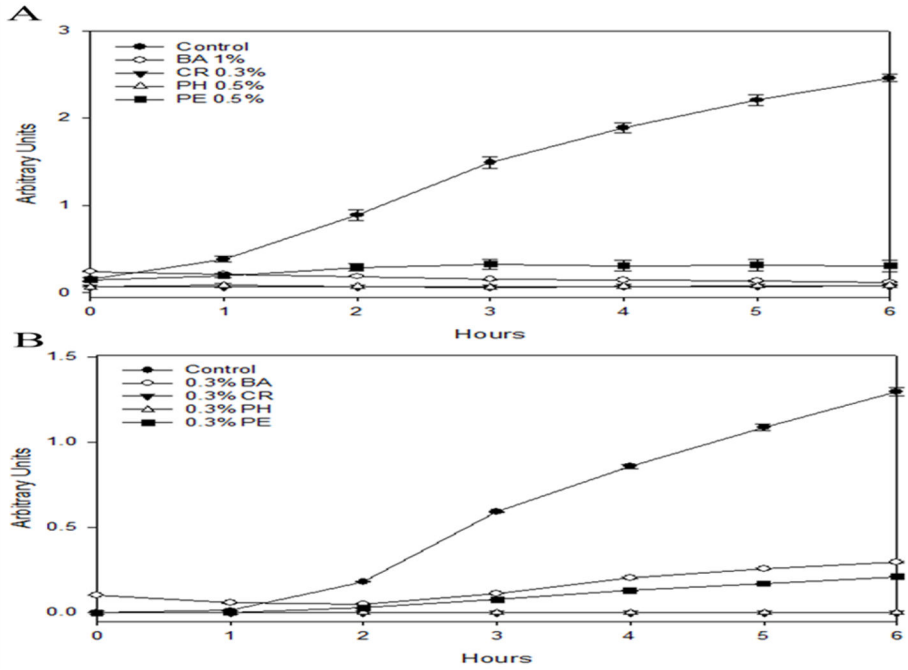


Figure 2.

Preservative efficacy test of APs on BL21(DE3) *E. coli* cells (Sutton and Porter 2002, Hutchings et al. 2013). The cell count was monitored by measuring the relative optical density at 600 nm as a function of the growth time. Addition of APs inhibited bacterial growth. (A) Preservative efficacy at the AP concentrations used in protein formulations. Closed circles represent the data untreated with APs, while the other symbols represent the data treated with various APs: 1% v/v benzyl alcohol (BA, open circles), 0.3% v/v m-cresol (CR, closed triangles), 0.5% v/v phenol (PH, open triangles), and 0.5% v/v phenoxyethanol (PE, closed squares), respectively. (B) Preservative efficacy at 0.3% v/v AP concentrations. Closed circles, untreated control; open circles, BA; closed triangles, CR; open triangles, PH; closed squares, PE.

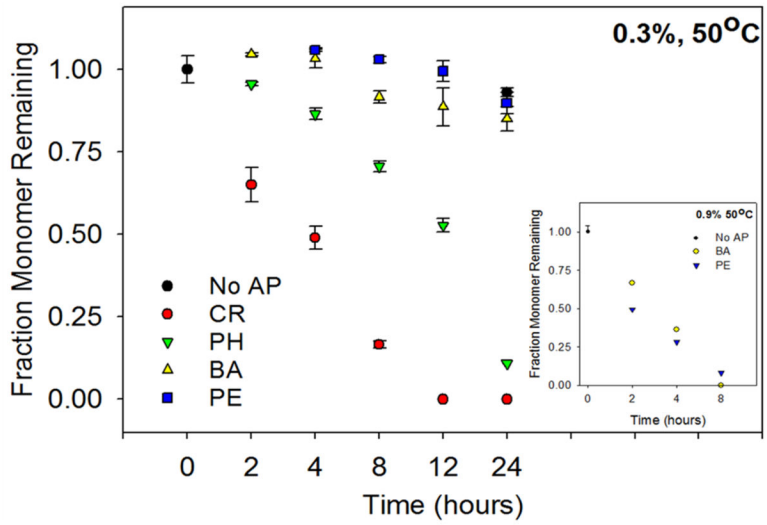


Figure 3.

Effects of APs on IFNA2 monomer concentration under isothermal conditions. Soluble monomer remaining in solution during 24 hours of incubation at 50°C in the presence and absence of 0.3% (v/v) AP as measured by size exclusion chromatography. Black – native (no AP); red – m-cresol (CR); green – phenol (PH); yellow – benzyl alcohol (BA); blue – phenoxyethanol (PE). Error bars indicate triplicate data. Individual time points are normalized with respect to the sample with no AP at hour zero.

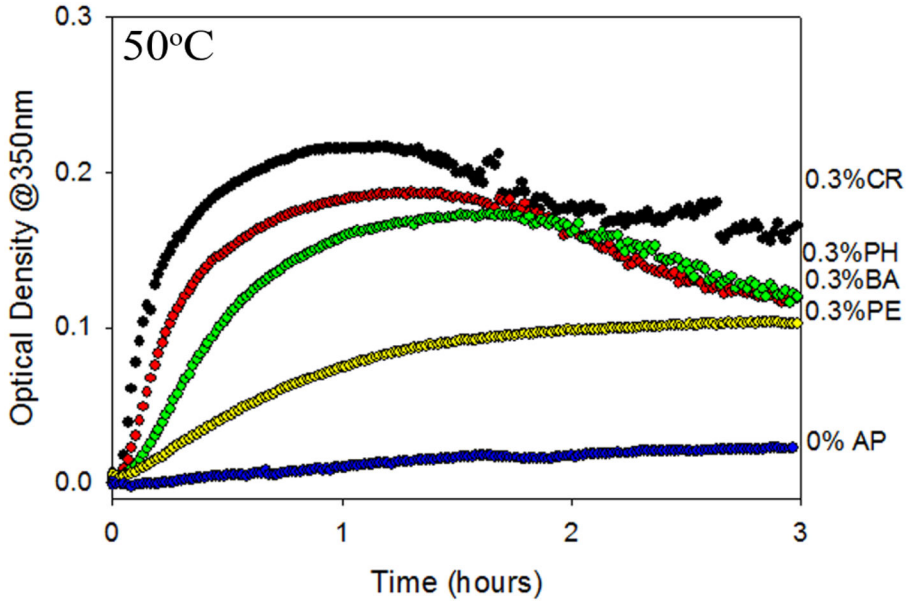


Figure 4.

Isothermal kinetics of IFNA2 aggregation at 50°C as measured by the changes in optical density at 350 nm in the presence of 0.3% v/v APs.

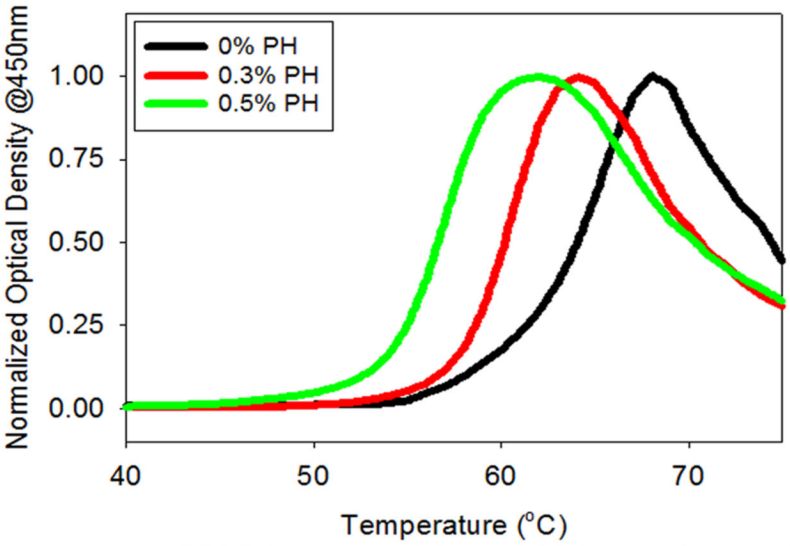


Figure 5.

Variation in the optical density at 450 nm as a function of increasing solution temperature at different concentrations of phenol (PH).

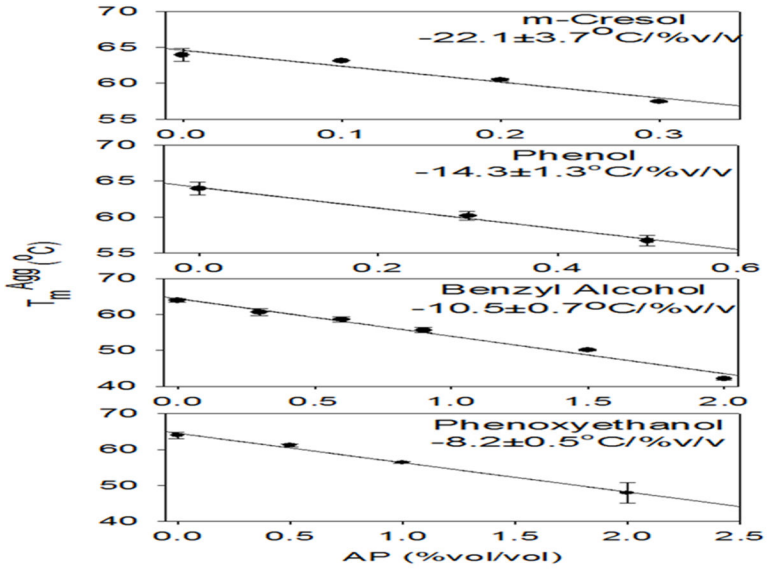


Figure 6.

Dependence of aggregation temperature T_m^{Agg} on AP concentration. Individual panels indicate the slope of this variation.

Table 1

Antimicrobial preservatives (AP) used in this study.

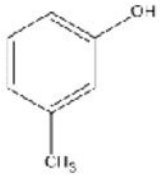
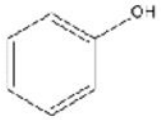
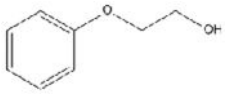
AP and Typical Use Concentration	Molecular Structure	Molecular weight (Da)	Source	Purity
Benzyl alcohol (BA) 1% vol/vol		108.1	Merck	97%
m-Cresol (CR) 0.3% vol/vol		108.1	Sigma	99%
Phenol (PH) 0.5% vol/vol		94.1	Sigma	99.5%
2-phenoxyethanol (PE) 1% vol/vol		138.2	Fluka	99.5%

Table 2

Minimum inhibitory concentration (MIC) values of APs measured using bacterial and microbial organisms (Lucchini et al. 1990, Simpson and Wuthiekanun 2000, Meyer et al. 2007, Rowe et al. 2009, Abd-Elsalam et al. 2011).

Preservative and Typical Use Concentration	Typical Use Concentration [μg/ml]	Organism Tested	MIC [μg/ml]
Benzyl Alcohol, 1% vol/vol	10400	Aspergillus niger Candida albicans Escherichia coli Pseudomonas aeruginosa Staphylococcus aureus	5000 2500 2000 2000 25
m-Cresol, 0.3% vol/vol	3090	Aspergillus niger Bacillus subtilis Burkholderia pseudomallei Candida albicans Pseudomonas aeruginosa Staphylococcus aureus	2000 1000 125 2500 1000 1000
Phenol, 0.5% vol/vol	5350	Aspergillus niger Bacillus aerogenes Enterococcus faecium Escherichia coli Pseudomonas aeruginosa Staphylococcus aureus	311 697 3600 2500 1800 1800
Phenoxyethanol, 1% vol/vol	11000	Aspergillus niger Candida albicans Escherichia coli Pseudomonas aeruginosa Staphylococcus aureus	3300 5400 3600 3200 8500