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Viscosity-reducing Bulky-salt Excipients Prevent Gelation of Protein, but not Carbohydrate, Solutions

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Abstract

The problem of gelation of concentrated protein solutions, which poses challenges for both downstream protein processing and liquid formulations of pharmaceutical proteins, is addressed herein by employing previously discovered viscosity-lowering bulky salts. Procainamide-HCl and the salt of camphor-10-sulfonic acid with L-arginine (CSA-Arg) greatly retard gelation upon heating and subsequent cooling of the model proteins gelatin and casein in water: whereas in the absence of additives the proteins form aqueous gels within several hours at room temperature, procainamide-HCl for both proteins and also CSA-Arg for casein prevent gel formation for months under the same conditions. The inhibition of gelation by CSA-Arg stems exclusively from the CSA moiety: CSA-Na was as effective as CSA-Arg, while Arg-HCl was marginally or not effective. The tested bulky salts did not inhibit (and indeed accelerated) temperature-induced gel formation in aqueous solutions of all examined carbohydrates — starch, agarose, alginate, gellan gum, and carrageenan.

Keywords Carbohydrates · Downstream processing of biologics · Gel formation · Hydrophobic salts · Intermolecular interactions in solution · Proteins

Introduction

Protein and peptide therapeutic agents continue to grow in importance and impact [1]. Since these biologics are often required at high concentrations in their pharmaceutical formulations, their manufacturing and downstream processing frequently encounter daunting challenges, such as high viscosity, aggregation, phase separation, and gelation [2-7]. In particular, gel formation in protein/peptide solutions, triggered by physical or chemical changes, has been recognized as a serious problem [7-9]. For example, aqueous solutions of human IgM cryoglobulin and IDEC-152 antibodies have been reported to readily undergo gelation caused by changes in temperature or pH [10, 11].

Recently, we reported that certain bulky hydrophobic organic salts can drastically lower the viscosity of concentrated aqueous solutions of various proteins [12, 13], as well as of DNA [14]. In the case of proteins, these charged hydrophobic excipients compete with non-covalent protein-protein interactions, thereby disrupting the putative transient protein networks responsible for solution's resistance to flow and hence lowering its viscosity [13].

In the present study, we have examined whether representative of the previously discovered by us viscosity-lowering bulky-salt excipients, namely procainamide-HCl and the salt of camphor-10-sulfonic acid with L-arginine (CSA-Arg), also affect the gelation of aqueous solutions or suspensions of such biomacromolecules as several proteins and carbohydrates. It has been found that these excipients indeed prevent, or at least drastically retard, the gel formation upon cooling of heated suspensions of the model proteins gelatin and casein; however, they do not inhibit (and indeed promote) the gelation of the model carbohydrates starch, agarose, sodium alginate, gellan gum, and carrageenan in the same temperature-change protocol.

Materials and Methods

Materials

Bovine skin gelatin (type B), cow's milk casein, potato starch, brown algae sodium alginate, gellan gum, seaweed carrageenan, camphor-10-sulfonic acid (CSA), L-arginine (Arg), Arg-HCl, and procainamide-HCl were all obtained from Sigma-Aldrich Chemical Co. Agarose was from Alfa Aesar and CSA-Na from Santa Cruz Biotechnology. All these reagents were used without further purification. The CSA-Arg salt was prepared as described by us previously [13].

Preparation of Gels

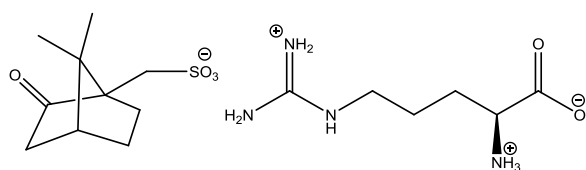
Both protein and carbohydrate gels were prepared by heating their aqueous suspensions (except for Na alginate and gellan gum that were soluble in room-temperature water at the concentrations used) to, and incubating for a certain time period at, a given elevated temperature, followed by gradual cooling to $24\pm 2^{\circ}\text{C}$ using a water bath. The elevated temperatures (guided by the cited literature) and the times of incubation thereat (optimized by us experimentally) were, respectively, as follows: gelatin, 40°C and 30 min [15]; casein, 100°C and 30 min [16]; starch, 100°C and 30 min [17]; sodium alginate, 70°C and 30 min [18]; gellan gum, 80°C and 30 min [19]; carrageenan, 80°C and 60 min [20]; and agarose, 100°C and 30 min [21].

Our operational definition of the time required to form a gel (always measured at least in duplicate) was when the corresponding aqueous mixture following heating and subsequent cooling remained undisturbed when the test tube containing it was inverted.

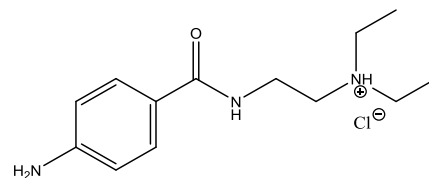
Results and Discussions

Bovine skin gelatin (a single polypeptide chain of collagen), a quintessential gel-forming protein of wide utility in food and pharmaceutical industries [22], was selected as the initial model in this study. It was suspended in water at 30 mg/ml, heated to 40°C, and incubated at that temperature for 30 min, followed by cooling to room temperature and monitoring the physical state of the resultant mixture as a function of time. As seen in Table 1, the aqueous solution turned into a transparent gel within 3 to 4 h in the pH range from 4.2 to 8.0.

As seen from their chemical structures depicted below, CSA-Arg and procainamide-HCl are bulky hydrophobic salts that, among others, were previously found to lower the viscosities of



CSA-Arg



procainamide-HCl

concentrated aqueous solutions of several monoclonal antibodies [13, 23]. Since the gelation of gelatin observed by us, in agreement with the literature [24], was preceded by a marked rise in solution viscosity, we reasoned that the bulky salts also might inhibit the gel formation. Indeed, 0.25 M CSA-Arg was found to afford a multi-fold retardation of gelatin's gelation at all the pH values examined (Table 1).

In order to ascertain whether CSA-Arg's constituent anion or cation is primarily responsible for this inhibitory effect, we separately examined the influence of 0.25 M CSA-Na and 0.25 M Arg-HCl on the time required to form a gelatin gel at different pH values. The data in Table 1 show that while CSA-Na was nearly as potent as CSA-Arg in inhibiting the gelation,

Arg-HCl exerted no significant influence. Since inspection of the CSA-Arg's chemical structure above reveals that most of its bulkiness and hydrophobicity stems from the CSA anion, it appears that the additive's ability to disrupt the intermolecular hydrophobic interactions among polypeptide chains [22] plays the major role.

A far more dramatic gelation-retarding effect was observed in the case of 0.25 M procainamide-HCl: as seen in Table 1, no gel formation was detected after months of incubation at room temperature following heating and cooling irrespective of the pH. Furthermore, no great thickening of the gelatin solutions occurred in the presence of this excipient either. These results indicate that procainamide is more effective than CSA in disrupting intermolecular hydrophobic interactions responsible for the formation of three-dimensional networks, and of a permanent gel, in gelatin's aqueous solutions [22]. The lack of any substantial and/or systematic pH dependence (Table 1) points to a minimal role of electrostatic interactions.

To test the generality of the foregoing findings, we next examined the gelation of a protein structurally and functionally unrelated to gelatin, namely of cow's milk casein [25, 26]. Following heating of a 200 mg/ml casein in water at 100°C for 30 min and subsequent cooling of the resultant mixture to room temperature, a gel formed within about 4 h. In stark contrast, in the presence of 0.25 M concentrations of procainamide-HCl or CSA-Arg no gelation was observed even after 4 months. As seen in Table 2, CSA-Na was as strikingly effective in preventing casein's gelation as CSA-Arg, while Arg-HCl merely afforded a 6-fold retardation effect. Therefore, as with gelatin (Table 1), bulky hydrophobic ions greatly inhibit gel formation, in this case apparently by disrupting hydrophobic interactions among casein micelles [25, 27].

Finally, we have explored the effect of bulky hydrophobic salts on gel formation induced by heating and subsequent cooling of aqueous solutions of a distinct class of biopolymers, namely carbohydrates. As seen in Table 3, 0.25 M procainamide-HCl or CSA-Arg not only fail to retard, but actually substantially accelerate, gel formation of starch, agarose, sodium alginate, gellan gum, and carrageenan. Unlike in proteins, the gelation of carbohydrates in water is chiefly due to intermolecular hydrogen bonding among their abundant hydroxyl groups [28, 29]. Presumably procainamide-HCl and CSA-Arg do not compete for those hydrogen-bond crosslinks and somehow even favor them.

Hence bulky hydrophobic salts alleviate phenomena originating in intermolecular hydrophobic interactions of biopolymers, such as high viscosity of concentrated aqueous solutions of proteins [12, 13] and DNA [14], as well as protein gelation described in this paper, but are ineffective in counteracting those due to intermolecular hydrogen bonding.

Conclusion

Bulky salts are found to drastically slow down the gel formation rates of proteins, but not of carbohydrates, in water upon heating and subsequent cooling; this phenomenon is mechanistically rationalized based on the dominant intermolecular interactions in aqueous solutions of the biopolymers.

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Table 1 The effect of excipients on gel formation of 30 mg/ml gelatin in aqueous solution at 25°C and various pH^a values.

pH	Excipient (0.25 M)	Time required to form a gel
4.2	None	3.5 ± 0.5 h
	CSA-Arg	65 h
	CSA-Na	65 h
	Arg-HCl	4.0 ± 0.5 h
	Procainamide-HCl	>105 days
5.2	None	3.5 ± 0.5 h
	CSA-Arg	23 h
	CSA-Na	18 h
	Arg-HCl	4.5 ± 0.5 h
	Procainamide-HCl	>141 days
7.0	None	3.5 ± 0.5 h
	CSA-Arg	52 h
	CSA-Na	51 h
	Arg-HCl	4.5 ± 0.5 h
	Procainamide-HCl	>113 days
8.0	None	3.5 ± 0.5 h
	CSA-Arg	24 h
	CSA-Na	26 h
	Arg-HCl	4.0 ± 0.5 h
	Procainamide-HCl	>102 days

^aThe pH was measured following heating and subsequent cooling before the gel formed. Due to high viscosities of the aqueous solutions of gelatin at that point, difficulties in pH adjustment resulted in deviations from the indicated pH values of up to ±0.2 pH units.

Table 2 The effect of excipients on gel formation of 200 mg/ml casein at 25°C and pH 6.6^a.

Excipient (0.25 M)	Time required to form a gel
None	4.0 ± 0.5 h
CSA-Arg	>141 days
CSA-Na	>141 days
Arg-HCl	24 h
Procainamide-HCl	>141 days

^a The pH was measured following heating and subsequent cooling before the gel formed. Due to extremely high viscosities of the aqueous solutions of casein at that point, difficulties in pH adjustment resulted in deviations from the indicated pH values of up to ±0.2 pH units.

Table 3 The effect of CSA-Arg and procainamide-HCl on gel formation of carbohydrates in aqueous solution at 25°C.

Carbohydrate	pH^a	Excipient (0.25 M)	Time required to form a gel (h)
Starch (200 mg/ml)	5.2	None	3
		CSA-Arg	0.5
		Procainamide-HCl	0.5
Sodium alginate (45 mg/ml)	7.0	None	1
		CSA-Arg	0.5
		Procainamide-HCl	0.5
Gellan gum (10 mg/ml)	6.0	None	4
		CSA-Arg	1
		Procainamide-HCl	1
Carrageenan (5.3 mg/ml)	7.8	None	4
		CSA-Arg	0.5
		Procainamide-HCl	0.5
Agarose (3.5 mg/ml)	7.0	None	0.5
		CSA-Arg	0.3
		Procainamide-HCl	0.3

^a The pH was measured following heating and subsequent cooling before the gel formed. High viscosities of aqueous solutions of the carbohydrates at that point resulted in deviations from the indicated pH values of up to ± 0.2 pH units.