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# Prevention of Thermally Induced Aggregation of IgG Antibodies by Noncovalent Interaction with Poly(acrylate) Derivatives

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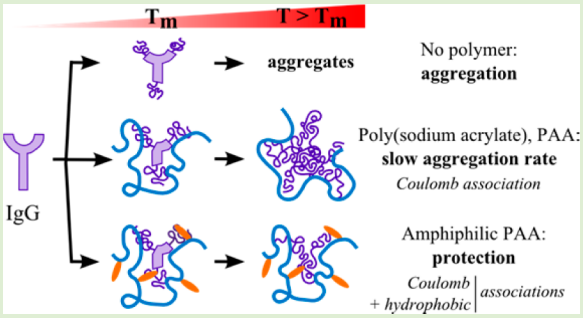
Supporting Information

**ABSTRACT:** Prevention of thermal aggregation of antibodies in aqueous solutions was achieved by noncovalent association with hydrophobically modified poly(acrylate) copolymers. Using a polyclonal immunoglobin G (IgG) as a model system for antibodies, we have studied the mechanisms by which this multidomain protein interacts with polyanions when incubated at physiological pH and at temperatures below and above the protein unfolding/denaturation temperature, in salt-free solutions and in 0.1 M NaCl solutions. The polyanions selected were sodium poly(acrylates), random copolymers of sodium acrylate and *N*-*n*-octadecylacrylamide (3 mol %), and a random copolymer of sodium acrylate, *N*-*n*-octylacrylamide (25 mol %), and *N*-isopropylacrylamide (40 mol %). They were derived from two poly(acrylic acid) parent chains of $M_w$ 5000 and 150000 g·mol$^{-1}$. The IgG/polyanion interactions were monitored by static and dynamic light scattering, fluorescence correlation spectroscopy, capillary zone electrophoresis, and high sensitivity differential scanning calorimetry. In salt-free solutions, the hydrophilic PAA chains form complexes with IgG upon thermal unfolding of the protein (1:1 w/w IgG/PAA), but they do not interact with native IgG. The complexes exhibit a remarkable protective effect against IgG aggregation and maintain low aggregation numbers (average degree of oligomerization <12 at a temperature up to 85 °C). These interactions are screened in 0.1 M NaCl and, consequently, PAAs lose their protective effect. Amphiphilic PAA derivatives (1:1 w/w IgG/polymer) are able to prevent thermal aggregation (preserving IgG monomers) or retard aggregation of IgG (formation of oligomers and slow growth), revealing the importance of both hydrophobic interactions and modulation of the Coulomb interactions with or without NaCl present. This study leads the way toward the design of new formulations of therapeutic proteins using noncovalent 1:1 polymer/protein association that are transient and require a markedly lower additive concentration compared to conventional osmolyte protecting agents. They do not modify IgG permanently, which is an asset for applications in therapeutic protein formulations since the in vivo efficacy of the protein should not be affected.




## INTRODUCTION

Therapeutic proteins, including antibodies, enzymes, or growth factors, play an increasingly important role in the treatment of viral infections, autoimmune disorders, cancers, and several other human diseases. Their medical use and commercialization are predicted to increase rapidly over the next few years. Together with the excitement generated by the efficacy of therapeutic proteins come concerns related to their biological half-life, insufficient stability, and aggregation.[1,2] Several approaches to enhance the stability of therapeutic proteins are actively pursued, either by tailoring the sequence of antibodies[2] or by chemical modifications.[3,4] Covalent attachment of water-soluble macromolecules, such as poly(ethylene glycol) (PEG), shields the proteins from their environment, which can improve the proteins stability in vivo, decrease their immunogenicity, and prolong their plasma half-life.[3−5] These beneficial factors are mitigated by the commonly observed

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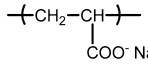
decrease in biological activity associated with protein PEGylation.

Noncovalent binding of proteins to synthetic molecules is another approach explored to enhance the stability of proteins. Currently, most proteins are formulated in solutions containing osmolytes, such as polyols[6] or arginine,[7] added in molar amounts and neutral detergents at micellar concentration. These additives must be added in amounts largely exceeding the 1:1 w/w protein/additive ratio, (e.g., 1 mol·L$^{-1}$ osmolytes or >CMC for detergents such as Tween).[8,9] Noncovalent association with polymers can also protect proteins against denaturation and aggregation. For instance, K. Akiyoshi et al. have shown that enzymes immobilized in pullulan nanogels are protected against thermal denaturation.[10] Mixtures of enzymes with poly(propylene oxide)[11] or poly(*N*-isopropylacrylamide)[12] were also investigated for similar purposes. Poly(4-styrenesulfonate) and poly(vinylsulfate) prevent the aggregation of denatured Cytochrome *c* near its isoelectric point by a mechanism involving Coulomb interactions.[13] Binding of polymers to proteins is the consequence of a complicated combination of hydrophobic attraction,[13−16] ionic bridges, and osmotic effects arising upon counterions release into bulk water,[17−20] hydrogen bonds, and steric contribution.[17,21] Since many interactions are involved, there are no accepted "rules" governing the design of synthetic polymers able to "cap" and protect proteins. For example, the stability of proteins has been reported to be enhanced[17,22] or decreased[23] upon association with polyelectrolytes of opposite charges via Coulomb interactions. There are no reports so far of the noncovalent polymer-induced stabilization of antibodies or antibody fragments, which is the objective of this study.

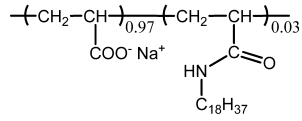
We report here an original approach to enhance the stability and bioavailability of antibodies, which relies on the formation of dynamic complexes between a protein and a water-soluble polymer of suitable chemical composition. Steric and electrostatic repulsion between the transient polymer/protein complexes is expected to play the same protective role as a polymer chain covalently linked to the protein. The labile nature of the polymer/protein association is likely to circumvent the deleterious effect of conjugated polymers on the protein bioactivity. Moreover, tailoring the polymer should allow one to achieve protein stability with a ∼1:1 w/w protein/polymer ratio, a value much lower than in the case of formulations using low molecular weight detergents. Polyclonal immunoglobulin G (IgG) was used here as a model antibody. IgG is a 150 kg mol$^{-1}$ multidomain protein, comprising four peptide chains connected by several S−S bonds, a complexity that makes IgG denaturation usually irreversible. IgG unfolds stepwise and generally undergoes parallel (irreversible) aggregation when heated above ∼60 °C. The polymers employed are presented in Scheme 1. They are poly(acrylates) differing in molar mass (5 or 150 kg mol$^{-1}$) and in composition. In addition to the homopolymers PAA5 and PAA150, we selected two copolymers of acrylate and *N*-*n*-octadecylacrylamide of different molar mass but identical composition (3 mol % *N*-*n*-octadecylacrylamide, PAAx-3C18) and a random terpolymer of acrylate, *N*-*n*-octylacrylamide (25 mol %), and *N*-isopropylacrylamide (40 mol %; PAA5−25C8−40C3). (The polymer also called amphipol "A8−35" is supplied by Affymetrix.) In a brief earlier report, we established that some of these polymers interact with IgG.[24] Coulomb interactions were invoked as the driving force to complexation with, possibly, a contribution of hydrophobic interactions in the case of copolymers of acrylate and *N*-alkyl-acrylamides. The mechanism of the interactions and their role in preventing protein aggregation are presented here.

**Scheme 1. Chemical Structures of the Polymers Investigated**[a]

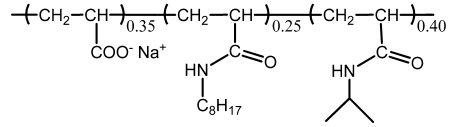
PAA*x* (*x* = 5 or 150)



PAA*x*-3C18 (*x* = 5 or 150)



PAA5-25C8-40C3



[a]PAA5-25C8-40C3 is also called A8-35 (trade name), but the notation PAA*x*-y*C*n was kept for consistency for all PAA chains (parent chain $M_w$ of "*x*" kg mol$^{-1}$, randomly modified with *y* mol % of *n*-alkyl side groups).

The formation of complexes between IgG and poly(acrylates) was monitored by static and dynamic light scattering (SLS and DLS), fluorescence correlation spectroscopy (FCS) using a fluorescently labeled IgG, and capillary zone electrophoresis (CZE). The thermal stability of IgG in the presence of polymers was assessed by differential scanning calorimetry (DSC), complemented by temperature-dependent SLS, DLS, and FCS studies. An important outcome of the study is the demonstration that in salt-free solutions Coulomb interactions are sufficient to retard markedly the heat-induced aggregation and that the amphiphilicity of polyacrylates helps preventing IgG aggregation in solutions of physiological ionic strength. The minimum amount of polymer required is in the micromolar range (in terms of polymer chains), which corresponds to an ∼1:1 w/w polymer/IgG ratio.

## EXPERIMENTAL SECTION

**Materials.** Water was deionized with a Milli-Q Millipore purification system. ChromPure Human IgG (Code No. 009−000−003) was purchased from Jackson ImmunoResearch Laboratories Inc. (as a 11.1 mg.mL$^{-1}$ stock solution). Poly(acrylic acid) (PAA) samples (nominal average molecular weight ($M_w$) 5000 g·mol$^{-1}$ (PAA5) or 150000 g·mol$^{-1}$ (PAA150)) were purchased from Sigma-Aldrich. The molar mass ($M_w$) and polydispersity index (PI) of the polymers in the sodium acrylate form measured by GPC using a Waters system equipped with Waters 2414 refractive index and Waters 2487 UV−visible absorbance detectors, and Waters styragel HR 5E (four columns) eluted with 0.5 M $LiNO_3$ were $M_w$ = 6500 g·mol$^{-1}$, PI ∼ 2 (PAA5) and $M_w$ ∼ 130000 g·mol$^{-1}$, PI ∼ 4 (PAA150) based on a calibration curve using poly(ethylene oxide) standards.[25,26] Random copolymers of acrylic acid and *N*-*n*-octadecylacrylamide (3−4 mol %, PAA5−3C18 and PAA150−3C18) were prepared by coupling *N*-*n*-octadecylamine to carbodiimide-activated PAA, following a procedure described previously.[26a] Analysis of the polymers demonstrated that this procedure gives evenly distributed alkyl modification and no residual unmodified PAA.[26b] PAA5−25C8−40C3 ($M_w$ 9000−10000 g·mol$^{-1}$) was purchased from ANATRACE (Amphipol A8−35, Affymetrix). Fluorescein isothiocyanate (FITC) was purchased from Fluka. The rhodamine-labeled polymers Rho-PAA150 and Rho-PAA150-3C18 were prepared as described in SI.

**Preparation of FITC-Labeled IgG.** A solution of IgG (2 mg·mL$^{-1}$) in a 0.5 M $NaHCO_3$−$Na_2CO_3$ buffer pH 9.5 was dialyzed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against the same buffer for 1 h. An aliquot of a freshly prepared FITC solution in anhydrous DMSO (5 mg·mL$^{-1}$) was added to the IgG solution (50 $\mu$g FITC/1 mg of IgG) to reach a final ratio of FITC/IgG of about 20:1 mol/mol). The reaction mixture was kept at room temperature for 2 h. It was dialyzed against sodium phosphate buffer saline (PBS: 20 mM phosphate, pH 6.8, NaCl 100 mM) for 1 h to remove unreacted FITC. The dialysate was purified by GPC using a Sephacryl 300 HR resin (Sigma-Aldrich) eluted with PBS. The concentration of FITC-IgG in the collected fractions was assessed by UV−vis spectrometry according to the relationship: [IgG] (mg·mL$^{-1}$) = ($A_{280} - 0.31 \times A_{495}$)/1.4 (with $A_{280}$ and $A_{495}$ being the absorbance at 280 and 495 nm, 0.31 the correction factor to account for the absorption of the dye at 280 nm, and 1.4 mL·mg$^{-1}$·cm$^{-1}$ the extinction coefficient of IgG). The molar ratio FITC/IgG (ca. 3:1) was determined from the following relationship: FITC/IgG = ($A_{495}$/69000)/([IgG] (mg·mL$^{-1}$)/150000) (with 69000 mol$^{-1}$·L·cm$^{-1}$ being the molar extinction coefficient of FITC at 495 nm (application note from Pierce FITC Antibody Labeling Kit, ThermoScientific) and 150000 g·mol$^{-1}$ the molar mass of IgG). Solutions were concentrated to ∼0.3 mg·mL$^{-1}$ by centrifugation using a Microcon centrifugal filter device (MWCO 100000).

**Preparation of IgG/Polymer Solutions.** Stock solutions of the polymers (2−20 mg·mL$^{-1}$) were prepared by dissolving freeze-dried polymer powders in deionized water under gentle stirring for at least 2 h at room temperature. The IgG solutions were used as received. Aliquots of concentrated buffer (0.5 M $NaH_2PO_4$/$Na_2HPO_4$, pH 6.8) and, in some cases, NaCl (0.5 M) were added to the polymer and IgG stock solutions to reach a final concentration of 20 mM phosphate and 100 mM NaCl. Solutions thus prepared with no added NaCl are referred to as "no NaCl", whereas solutions containing 100 mM NaCl are designated as "with NaCl". Prior to measurements, the IgG/polymer mixed solutions were dialyzed for at least 3 h against the appropriate buffer (Slide-A-Lyzer, MWCO 3500, from Pierce). The IgG concentration in the dialyzed solutions was determined from the solution absorbance at 280 nm, using the extinction coefficient of 1.4 mL·mg$^{-1}$·cm$^{-1}$ (Tech Tip #6, "Extinction Coefficients", ThermoScientific). The protein concentration was adjusted to ∼0.2 mg·mL$^{-1}$ by dilution in the dialyzing buffer.

**Differential Scanning Calorimetry.** DSC measurements were performed on a VP DSC microcalorimeter (MicroCal Inc.) with a cell volume of 0.520 mL and under an external pressure of about 180 kPa. The heating rate was set at 0.2 °C·min$^{-1}$ in the range of 20−95 °C. The experimental data were analyzed using the Origin 7.0 software supplied by the manufacturer. Solutions were kept at 20 °C for 1 h and degassed at 20 °C under mild vacuum for 15 min prior to loading into sample and reference cells. The reference cell was filled with the same buffer as the IgG and IgG/polymer solutions. All measurements were carried out in duplicates. A scan recorded with buffer in the sample and reference cells was subtracted from the sample data to remove baseline contributions. The molar enthalpy of the transition, $\Delta H$, was calculated using eq 1:

$$\Delta H = \int_{T_i}^{T_f} C_p(T)\cdot dT \tag{1}$$

where $C_p$ is the corrected excess heat capacity normalized by the concentration of protein, $T_i$ = 20 °C and $T_f$ = 90 °C.

**Fluorescence Correlation Spectroscopy.** FCS measurements were performed on a home-built two-photon excitation system equipped with a mode-locked Ti:sapphire laser (Mira900, Coherent, Auburn, CA) pumped by a solid-state laser at 532 nm (Verdi, Coherent). The laser beam (780 nm, ∼100 fs pulse width) was focused into the sample using a 60× water immersion microscope objective (1.2 NA, UPlanApo, Olympus). The power was kept below 10 mW by means of neutral filters. The fluorescence signal was collected through the same objective lens, reflected by a dichroic mirror to select fluorescein fluorescence (wavelength 580 ± 30 nm), and filtered through short pass filters to absorb any possible diffused excitation infrared light. The collected light was then separated by a beam splitter and focused on the 200 $\mu$m$^2$ working surfaces of two avalanche photodiode modules, APDs (SPCM-AQR-14, PerkinElmer, Vaudreuil, Canada). The signal outputs of the APDs (TTL pulses) were acquired by a digital autocorrelator module (ALV-6000, ALV-GmbH, Langen, Germany) which computed online the cross-correlation function of the fluorescence fluctuations, $g(t)$. The data were analyzed using the MEMFCS algorithm described elsewhere.[27] The diffusion time measured at the maximum of the distribution obtained with the MEMFCS algorithm was related to the diffusion coefficient by $\tau_D = \omega_{xy}^2/8D$. The hydrodynamic radius was then calculated via the Stokes−Einstein equation. The excitation area $\omega_{xy}^2$ was calibrated by using the diffusion coefficient of fluorescein in water at pH 10 as a standard.

IgG/polymer solutions were prepared in phosphate buffer with or without 100 mM NaCl, as described above, with both IgG and polymer concentrations of 0.2 mg·mL$^{-1}$. Each sample was heated at a rate of 0.2 °C·min$^{-1}$ from 25 to 85 °C. Aliquots (1 $\mu$L) of the sample were collected at various temperatures during the scan. They were diluted into 9 $\mu$L of buffer and kept at room temperature 10 min prior to fluorescence fluctuation recording (10 sets of 60 s acquisition).

The average number of IgG molecules per fluorescent object in solution ($N_{agg}$) was determined as follows. The limit of $g(t)$, the fluorescence correlation function, as $t$ approaches zero, $g_0$, is related to the apparent number, $n$, of fluorescent species in the experimental volume by $g_0 = 1/n$. The mean fluorescence intensity, $I$, is given by $I = N\cdot i$, where $N$ is the total number of fluorescent proteins (FITC-IgG) in the excited volume, and $i$ is the fluorescence intensity of a single FITC-IgG molecule. The average fluorescence intensity per object is $I\cdot g_0 = I/n = i\cdot N/n = i\cdot N_{agg}$, where $N_{agg}$ is the average number of IgG molecules per fluorescent object in the solution. This average number accounts for emission from free (unbound) FITC-IgG monomers, FITC-IgG complexes with polymers and FITC-IgG oligomers or aggregates. The $N_{agg}$ value can be determined using $i = I/N = (I\cdot g_0)_0$, where $(I\cdot g_0)_0$ is the value of $I\cdot g_0$ for FITC-IgG monomers in the absence of polymer at room temperature.

**Capillary Zone Electrophoresis (CZE).** CZE was performed using a Beckman P/ACE system MDQ instrument equipped with a diode array UV/visible detector (Beckman Instruments, Fullerton, CA), a working wavelength of 220 nm, operating at 25 °C, and fitted with bare silica capillaries of 75 $\mu$m × 31 cm, effective length of 21 cm (Chromoptic, France). The capillary was flushed daily with 1 M NaOH, followed by a water rinse, and finally was allowed to equilibrate with the running buffer (20 mM phosphate buffer pH 6.8 with or without 100 mM NaCl, with or without polymers). Solutions of IgG (3 mg.mL$^{-1}$) in the running buffer containing mesityl oxide as marker of the electro osmotic flow (EOF) were injected using the pressure injection mode (3 s at 0.3 psi). A voltage of 10 kV (respectively, 5 kV) was applied in the absence (respectively, presence) of 100 mM NaCl in running buffer. The mobility ($\mu$) was calculated from eq 2, where $t_{samp}$ is the sample migration time, $t_{EOF}$ is the migration time of the EOF marker, $L_t$ is the total length of the capillary, $L_d$ is the length to the detector, and $E$ is the electric field.

$$\mu = \frac{L_t\cdot L_d}{E}\cdot\left(\frac{1}{t_{samp}} - \frac{1}{t_{EOF}}\right) \tag{2}$$

**Static Light Scattering.** The aggregation kinetics of IgG with NaCl were assessed by measuring the intensity of the light scattered at a fixed angle of 90° using an ALV/CGS-3 Compact Goniomerer System equipped with a 22 mW HeNe laser operating at a wavelength of 632.8 nm. Toluene was used as the standard to normalize the intensity. Prior to measurement, the samples were filtered through Anotop10 filters (pore size: 20 nm, Whatman). The concentration of IgG after filtration was ∼0.5 mg·mL$^{-1}$, as determined by the solution absorbance at 280 nm using an extinction coefficient of 1.4 mL·mg$^{-1}$·cm$^{-1}$. The polymer/IgG ratio was ∼1:1 w/w. The scattering intensity recorded for solutions at room temperature prior to thermal treatment is defined as intensity at time 0. Subsequently, the IgG solutions were kept at a fixed temperature (55, 60, or 65 °C). Changes in the scattering intensity were measured as a function of incubation time at each of the three selected temperatures.

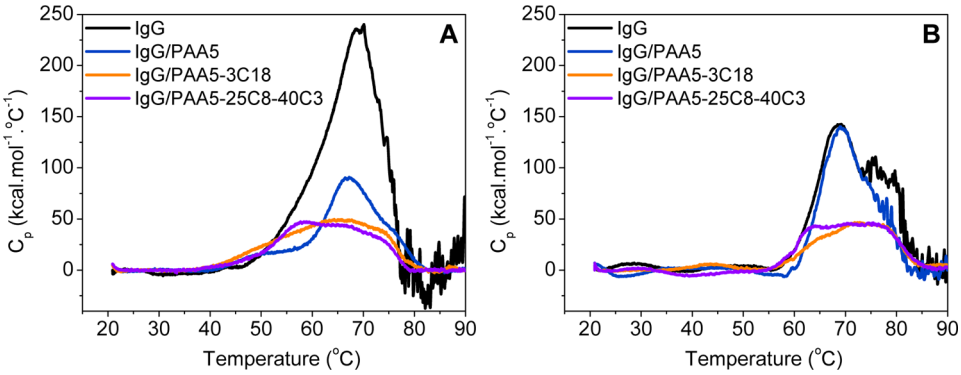


**Figure 1.** DSC thermograms (scan rate = 0.2 °C·min$^{-1}$) of phosphate-buffered (20 mM, pH 6.8) 1:1 w/w IgG/polymer mixtures at 0.2 mg·mL$^{-1}$ in the absence (A) or presence (B) of 100 mM NaCl.

## RESULTS AND DISCUSSION

**Calorimetric Study of Thermal Denaturation of IgG.** DSC thermograms of the thermal denaturation of IgG solutions of pH 6.8 and concentration of 0.2 mg·mL$^{-1}$ were recorded in the absence and presence of polymers. The reversibility of the unfolding process was checked by reheating the samples after they had been cooled to room temperature in the microcalorimeter. In all cases, denaturation or partial unfolding was irreversible (not shown). The thermogram of IgG in the absence of polymer (black line in Figure 1) consisted of a broad asymmetric peak spanning from ∼55 to 80 °C with a maximum at about 69 °C ($T_m$), in agreement with previous reports.[28−30] The shape of the endotherm is characteristic of the unfolding of multidomain proteins, such as IgG, which occurs in several overlapping steps that cannot be resolved by microcalorimetry. The polyclonal nature of IgG may also introduce a broadening of the peaks. Above 75 °C, the endotherm is quite noisy, an effect usually attributed to protein aggregation.[28] The cooled sample recovered after heating had a milky appearance (not shown), confirming the formation of large protein aggregates.[28,30]

Thermograms recorded for mixed solutions of IgG with either PAA5−3C18 or PAA5−25C8−40C3 in 20 mM phosphate buffer, pH 6.8 (no NaCl) were broad and featureless, spanning from ∼40 to 75 °C, with no well-defined maximum. Interestingly, the high temperature sections of the thermograms were smooth, suggesting that aggregation did not occur or, at least, that it took place to a much lesser extent than in the case of IgG alone. The enthalpy of the transition, taken as the area under the transition endotherm, for IgG in solution in the presence of either PAA5−3C18 or PAA5−25C8−40C3 was significantly lower than the enthalpy associated with the thermal denaturation of IgG alone in solution (see Table 1). Mixed IgG/polymer solutions recovered after heating were clear to the eye. UV-absorbance spectra of the solutions showed no sign of turbidity (see Figure S1 in SI). Amphiphilic polyanions, such as PAA5−3C18 or PAA5−25C8−40C3, can interact with IgG and protect it against thermal aggregation via hydrophobic interactions between hydrophobic domains of IgG and the alkyl chains of the polymer and also by electrostatic attraction. Poly(acrylic acid) (PAA), a polyanion bearing no hydrophobic side groups, triggered also a significant decrease in the transition enthalpy of IgG, with no sign of IgG aggregation (Figure 1A, Table 1, and Figure S1 in SI), which suggests that electrostatic interactions between PAA and IgG provide some protection against thermal aggregation and that hydrophobic association is not the sole type of interaction at play. The involvement of electrostatic interactions on the stability of IgG/PAA5 solutions was confirmed by assessing the effect of salt, known to screen ionic interactions. As seen in Figure 1B, the thermogram recorded for mixed IgG/PAA5 in a 100 mM NaCl solution presented all the signs of IgG aggregation. It was nearly identical to the thermogram of IgG alone. Similar to the case of IgG alone, the mixed IgG/PAA5 solution recovered after heating was turbid, as shown by the UV−visible absorption spectrum presented in Figure S2 in SI. In contrast, thermograms recorded for solutions of IgG and the amphiphilic polymers PAA5−3C18 and PAA5−25C8−40C3 containing 100 mM NaCl (Figure 1B) retained the characteristic features observed for the corresponding solutions without NaCl: the thermograms were broad, their $T_m$ was shifted to lower temperatures, and the enthalpies of the transitions were similar to those obtained for these mixtures in salt-free conditions (Table 1). Hence, IgG appears to be protected against aggregation by amphiphilic polymers under conditions for which PAA has no effect, confirming that hydrophobic interactions significantly strengthen the interactions of IgG with PAA5−3C18 or PAA5−25C8−40C3. Polymers of higher molecular weight (based on PAA150) had the same effects (see Figure S3 and Table S1 in SI).

**Table 1. Temperature and Enthalpy of Thermal Denaturation of 0.2 mg·mL$^{-1}$ IgG, in the Absence or Presence of Poly(acrylate) Derivatives (Added at 1:1 w/w)[a]**

| | $T_m$ (°C) | | $\Delta H$ (kcal·mol$^{-1}$) | |
|---|---|---|---|---|
| condition | no NaCl | 100 mM NaCl | no NaCl | 100 mM NaCl |
| no polymer | 69.5 | 68.9 | 3300 | 2200 |
| PAA5 | 67 | 68.9 | 1100 | 2000 |
| PAA150 | 67 | nd | 1050 | nd |
| PAA5−3C18 | 66 | broad | 900 | 700 |
| PAA150−3C18 | 64 | nd | 600 | nd |
| PAA5−25C8−40C3 | broad | broad | 1100 | 900 |

[a]Scan rate: 0.2 °C·min$^{-1}$; 20 mM phosphate buffer, pH 6.8, supplemented or not with 100 mM NaCl as quoted. When a shoulder or several peaks were observed, $T_m$ of the main peak is indicated; "broad": broad peak making it difficult to determine $T_m$; nd: not determined.

Next, we recorded the thermograms of IgG in the presence of increasing polymer amounts in order to determine the optimal polymer/IgG weight ratio. For solutions of IgG and either PAA5−3C18 or PAA5−25C8−40C3, both $T_m$ and $\Delta H$ decreased gradually with increasing polymer/IgG ratio (Figure


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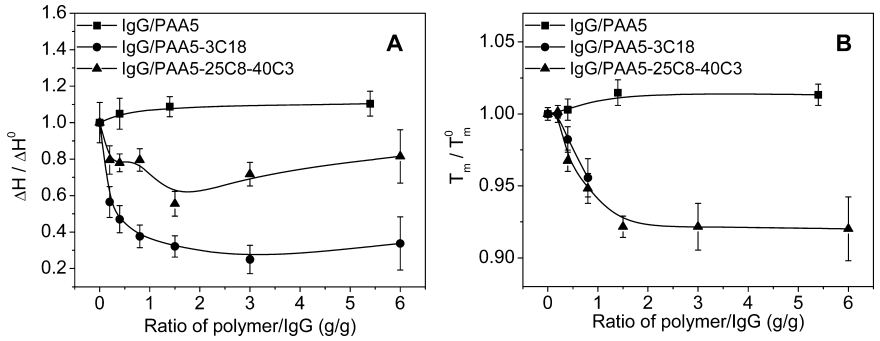


**Figure 2.** Variation of the enthalpy of thermal denaturation, $\Delta H$, and unfolding temperature, $T_m$, of IgG measured by DSC as a function of the polymer/protein weight ratio. The concentration of IgG was 0.3 mg·mL$^{-1}$. Lines are shown to guide the eye. All solutions were buffered in 20 mM sodium phosphate at pH 6.8 with 100 mM NaCl. With PAA5−3C18, $T_m$ could not be measured above the ratio of 1:1 g/g, because the shape of endotherms turned into several overlapping curves (see details in Figure S4 in SI).

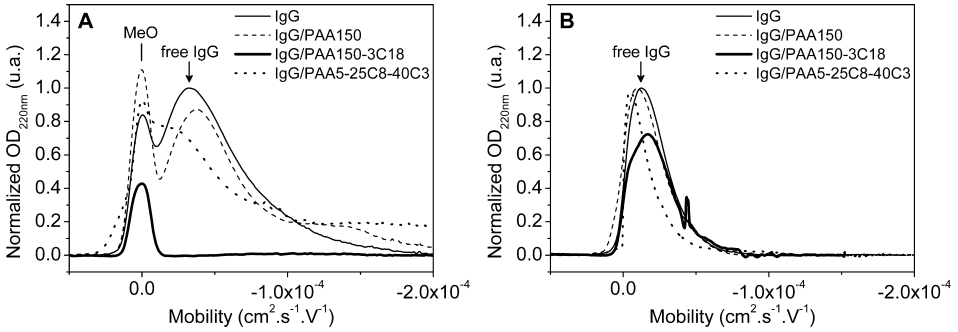


**Figure 3.** Electrophoretic mobility of native IgG in the presence of polymers in the 20 mM $NaH_2PO_4/Na_2HPO_4$, pH 6.8 running buffer: (A) 0.5 g·L$^{-1}$ polymer with no added NaCl; (B) in the presence of 100 mM NaCl and 0.5 g·L$^{-1}$ polymer. IgG stock solution was 3 mg·mL$^{-1}$ in the running buffer.

2, and Figure S4 and Tables S2−S4 in SI), reaching constant values for polymer/IgG ∼ 1.0−1.5 g/g. This value can be taken as the minimum amount of amphiphilic polymer needed to protect IgG against aggregation. It corresponds to a millimolar concentration of polymer repeat units or about 30 PAA5−25C8−40C3 chains per IgG. This polymer concentration is significantly lower than the concentration of osmolytes required to achieve the same effect.[2,6] Control experiments performed with IgG/PAA5 solutions (100 mM NaCl) indicated that thermal denaturation of IgG was not affected in the same range of experimental concentrations (<1.8 mg·mL$^{-1}$), which confirms the efficient screening of electrostatic interaction in these conditions.

From the DSC study, we conclude that all the polymers tested prevent the massive aggregation of IgG in the buffer at low ionic strength, but only amphiphilic polymers are efficient protective agents of IgG in the presence of 100 mM NaCl. The amphiphilic polymers also induce a decrease of the unfolding enthalpy, which is taken as an indication that polymers are tightly bound to the IgG with a binding enthalpy of magnitude similar to the enthalpy of folding.

**Detection of Polymer/IgG Complexes by Capillary Zone Electrophoresis.** To confirm that complexation between polymers and IgG can occur at room temperature, we carried out an analysis of mixed polymer/IgG solutions by capillary zone electrophoresis, a technique that yields the electrophoretic mobility of IgG in various solutions. Electropherograms of IgG alone and in the presence of polymers are presented in Figure 3. The mobility of IgG alone in NaCl-free buffer was $-3.2 \times 10^{-5}$ cm$^2$·s$^{-1}$·V$^{-1}$. It decreased to $-0.84 \times 10^{-5}$ cm$^2$·s$^{-1}$·V$^{-1}$ in solutions containing 100 mM NaCl. The signal of IgG used was broad compared to the signal of mesityl oxide (MeO, indicated by a line in Figure 3) used as internal neutral standard, probably as a consequence of the polydispersity of pI of the polyclonal IgG used in this study. The electropherogram of a mixed IgG/PAA150−3C18 (0.5 g·L$^{-1}$) solution in salt-free buffer presented no detectable signal due to free IgG (Figure 3A, thick line), but a signal was detected at a higher mobility, $\mu \approx -30 \times 10^{-5}$ cm$^2$·s$^{-1}$·V$^{-1}$, a value characteristic of highly charged polymers, such as polyacrylates in their sodium salt form.[18,31] The new signal was attributed to complexes between PAA150−3C18 and IgG (Figure S5 in SI). The formation of complexes with PAA5−3C18 was detected by similar CZE experiments (Figure S5 in SI). For NaCl-free solutions, the signal ascribed to unbound IgG progressively decreased with increasing PAA150−3C18 concentration (Figure S6A in SI). Adsorption isotherms derived from these measurements reach a plateau value for solutions with a w/w IgG/PAA150−3C18 ratio of 1:1 (Figure S6C in SI). Addition of 100 mM NaCl strongly decreased the affinity of PAA150−3C18 for native IgG (Figure S6B in SI). We note in Figure 3B a slight decrease in the intensity (<15%) of the IgG signal in 100 mM NaCl in the presence of PAA150−3C18.

When CZE experiments were carried out with IgG and the other polymers of this study, in the absence or presence of NaCl (100 mM), the corresponding electropherograms provided little evidence of IgG/polymer association at room temperature. Electropherograms of solutions of IgG in the presence of PAA150 or PAA5−25C8−40C3 (no NaCl) exhibited a signal due to free IgG that was slightly weaker


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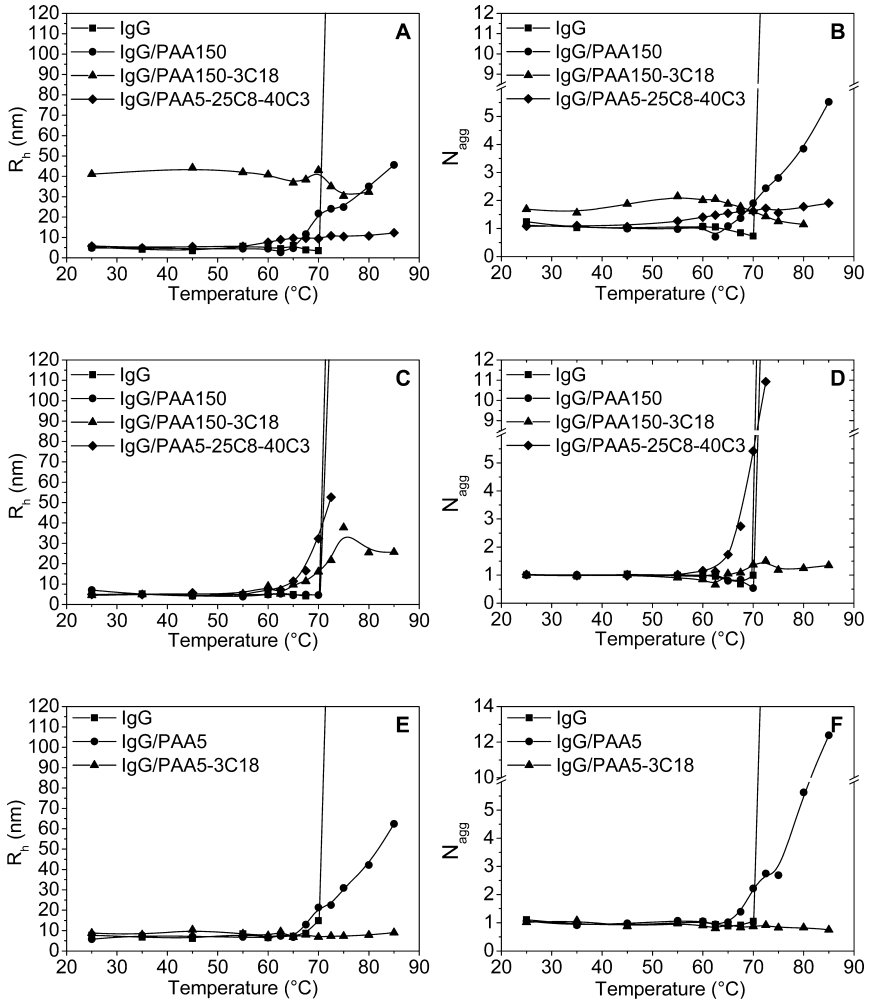


**Figure 4.** Variation upon incubation at increasing temperature of the hydrodynamic radius ($R_h$) and aggregation number of IgG ($N_{agg}$, defined as the averaged number of IgG molecules per fluorescent object in solution) measured by FCS in phosphate-buffer (20 mM, pH 6.8) at 1:1 w/w IgG/polymer ratio, 0.2 mg·mL$^{-1}$ IgG, (A, B) in the absence or (C, D) in the presence of 100 mM NaCl for the long polymers, and (E, F) in the absence of 100 mM NaCl for the short polymers.

than the signal of IgG alone and presented significant tailing. The tailing of the IgG peak in the presence of PAA150, PAA5, or PAA5−25C8−40C3 (Figure S5 in SI) may be ascribed to the formation of transient and weak interactions with IgG (in NaCl-free buffer). It was not observed in electropherograms of mixed polymers/IgG solutions containing NaCl. Keeping in mind that the concentration of IgG used in CZE experiments is about 10× higher than the concentration used in DSC measurements, we conclude that complexes with IgG at room temperature are most likely NOT formed in solutions used for DSC, except in the case of PAA150−3C18.

**Detection of Polymer/IgG Complexes and IgG Aggregates by FCS.** All FCS measurements were carried out with mixed solutions of a fluorescently labeled IgG (FITC-IgG) and unlabeled polymers used in equal weight, at a concentration of 0.2 mg·mL$^{-1}$. Since FCS measurements detect only fluorescent entities, single polymer chains or polymer aggregates are invisible under these conditions. Prior to thermal treatment, the hydrodynamic radius ($R_h$) of FITC-IgG alone in a buffer solution at room temperature was ∼5 nm, in agreement with values previously reported for the native IgG monomer.[7] The same $R_h$ value was recorded for room temperature solutions of FITC-IgG containing either PAA150, PAA5, or PAA5−25C8−40C3, independently of salt concentration, bringing further support to the conclusion derived from DSC and CZE studies that native IgG does not interact with these polymers at room temperature. The FITC-IgG $R_h$ value was also ∼5 nm in mixed FITC-IgG/PAA150−3C18 solutions containing 100 mM NaCl. However, much larger objects were observed in mixed solutions of FITC-IgG/PAA150−3C18 in the absence of NaCl. The $R_h$ of the fluorescent objects in this case was ∼40 nm, a size higher than the $R_h$ of PAA150−3C18 alone in the same buffer ($R_h$ ∼ 16 ± 3 nm from FCS measurement on rhodamine-labeled PAA150−3C18, see Figure S7 in SI), confirming that this polymer interacts with IgG at room temperature, presumably through a combination of hydrophobic and electrostatic interactions, in agreement with CZE results.

Next, the temperature-dependent aggregation status of FITC-IgG in mixed FITC-IgG/polymer solutions heated from 25 to 85 °C at a rate of 0.2 °C·min$^{-1}$ was evaluated by FCS. Figure 4 displays the temperature dependence of the hydrodynamic radius ($R_h$) and the number-averaged degree of aggregation ($N_{agg}$) of the fluorescent objects detected in solutions of FITC-IgG in phosphate-buffer pH 6.8, having a 1:1 w/w protein/polymer ratio. Incubation of the solutions was


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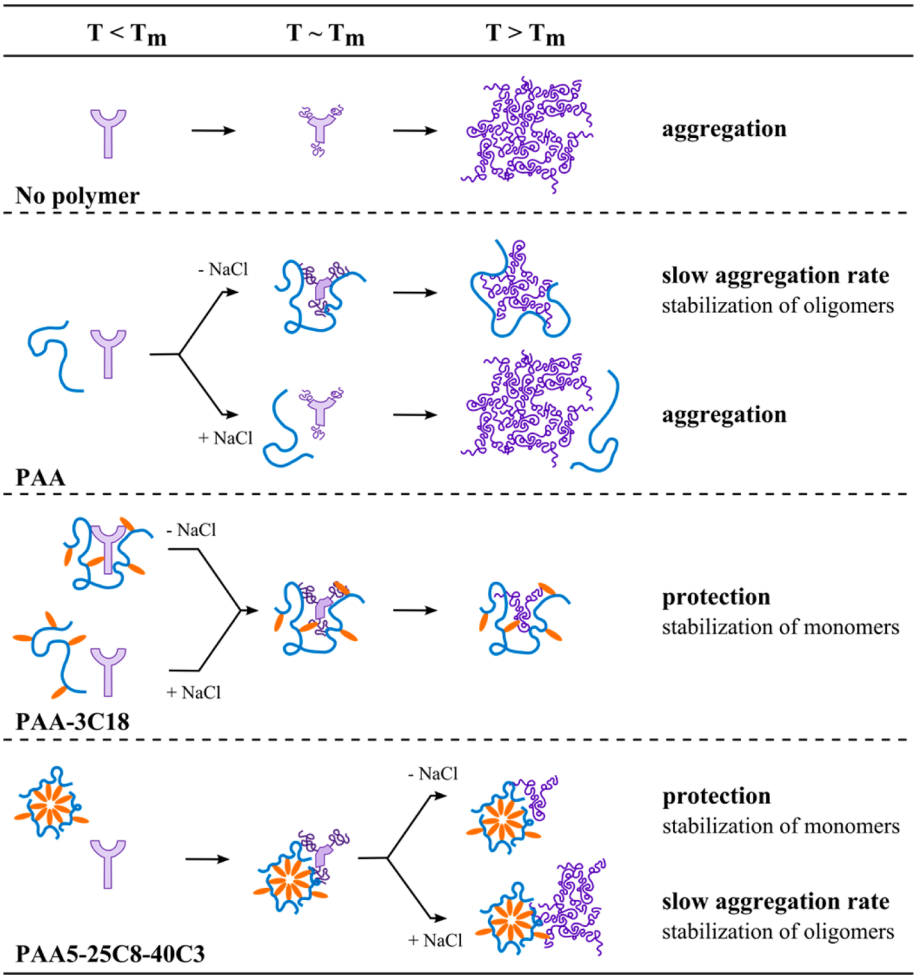
**Scheme 2. Schematic Summary Describing the Temperature-Dependent Interactions between IgG and Amphiphilic Polyanions, Derived from DSC, SLS, FCS, and CZE Results**




performed under conditions identical to those used in the DSC experiments reported in the previous section (0.2 mg·mL$^{-1}$ protein in the same buffer conditions and same heating rate of 0.2 °C·min$^{-1}$ between 25 and 85 °C). However, in view of the high sensitivity of FCS, aliquots taken at different temperatures had to be diluted by a factor of 10 prior to measurements. This was done by adding the aliquot to a solution of the same buffer kept at room temperature. Thus, the experiments described below report the sizes of objects intact after dilution and cooling.

Massive aggregation of FITC-IgG alone in solution occurred at ~70 °C, independent of the salt concentration (vertical lines in the plots of $R_h$ and $N_{agg}$ versus temperature in Figure 4). This temperature is close to the $T_m$ of IgG recorded by DSC (Figure 1). The presence of polymers had a remarkable effect on the colloidal stability of FITC-IgG, particularly in salt-free solutions (Figure 4A,B,E,F): in the absence of NaCl, both $R_h$ and $N_{agg}$ remained nearly constant up to a temperature (~60−65 °C) slightly below $T_m$ of IgG. Their value did not vary significantly beyond this temperature. In some cases, they changed gradually, confirming that IgG/polymer mixtures resist aggregation. In the case of mixed FITC-IgG/PAA5−3C18 solutions, $N_{agg}$ and $R_h$ remained constant upon heating up to 85 °C. In mixed FITC-IgG/PAA150−3C18, $N_{agg}$ and $R_h$ decreased slightly. In mixed FITC-IgG/PAA5−25C8−40C3 solutions they increased by a factor of about two, and in mixed FITC-IgG/PAA5 and FITC-IgG/PAA150, they increased by a factor of less than 10. The mixed FITC-IgG/PAA150−3C18 system stands out, likely reflecting the fact that IgG associates with PAA150−3C18 prior to denaturation, forming IgG/PAA150−3C18 complexes (~40 nm) that do not aggregate up to 80 °C. The FITC-IgG aggregation numbers derived from FCS analysis (Figure 4B,D,F), indicate that the protein exists in the monomeric form in all mixed solutions kept below ~65 °C, except in the case of mixed IgG/PAA150−3C18 solutions of low ionic strength, where IgG/polymers complexes, containing on average two proteins/polymer chain, form at all temperatures. In the other mixed systems heated above 65 °C that did not form aggregates exceeding the range of FCS, the IgG/polymer complexes contained from 3 to 10 proteins.

FCS measurements conducted with mixed polymer/IgG solutions containing 100 mM NaCl showed a marked dependence on polymer structure, as indicated by the values of $R_h$ and $N_{agg}$ (Figure 4C,D). In the case of FITC-IgG mixed with PAA150, the partial stabilization achieved in salt-free solutions, with a final $N_{agg}$ < 10 and final $R_h$ < 50 nm, was not effective in solutions with 100 mM NaCl. When the temperature reached 70 °C, the size of the fluorescent objects increased as sharply as in the case of IgG alone. This result strengthens our conclusion that IgG/PAA150 complexes formed in the absence of salt owe their stability solely to electrostatic interactions. In contrast, amphiphilic polymers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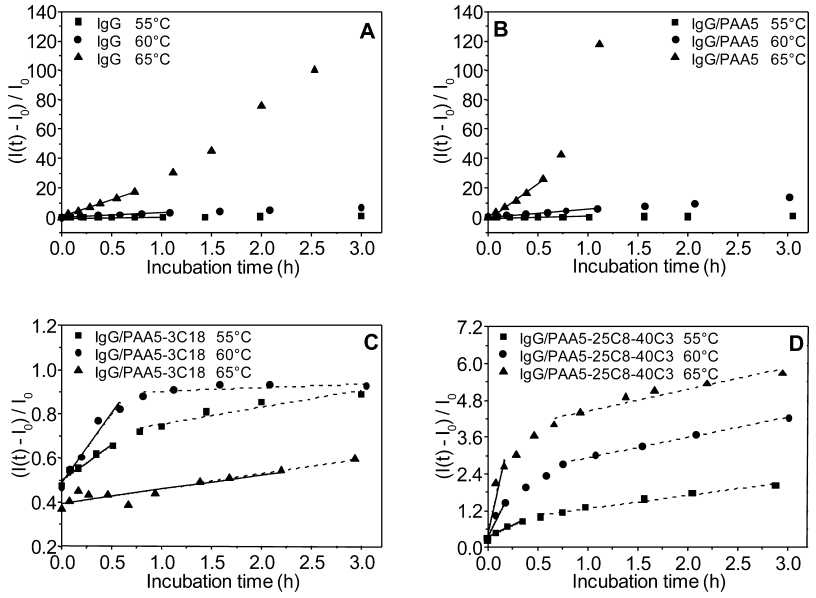


**Figure 5.** Variation of the normalized scattering intensity $(I(t) - I_0)/I_0$ as a function of incubation time. Solid lines correspond to the fits of data points at time <20−40 min to eq 3 (see Experimental Section); dotted lines correspond to linear fits of data points beyond 1 h incubation time.

kept their protective effect toward IgG in buffer solutions containing 100 mM NaCl. The $N_{agg}$ of IgG in the presence of PAA150−3C18 remained ∼1 (triangles in Figure 4D), indicating that this polymer prevented both thermal aggregation and oligomerization of IgG, independent of ionic strength (at least up to 100 mM NaCl). Interestingly, in solutions of FITC-IgG/PAA150−3C18 with 100 mM NaCl, the $R_h$ value increased, from about 5 nm below 60 °C up to ∼30 nm at 85 °C, implying the absence of complexes at low temperature and the formation of dilution-resistant polymer/protein complexes upon heating. Results from DLS measurements with short polymers were consistent with the FCS results (Figure S8 in SI), keeping in mind that it is not possible to distinguish by DLS the contributions of the polymers and of the IgG to the signal and to size distribution.

Overall, the experimental data reported so far yield the following ranking of the polymers in the order of their ability to limit thermal aggregation and oligomerization of IgG: PAA150−3C18 ≈ PAA5−3C18 > PAA5−25C8−40C3 > PAA150 ≈ PAA5 (the latter two polymers being efficient at low ionic strength only). A summary of the FCS results on the thermally induced complexation and aggregation of IgG in the absence/presence of polymer is given in Table S5 in SI and in Scheme 2.

**Temperature Dependence of the Initial Rate of Aggregation of IgG in the Presence of Polymers (SLS).** The changes with time of the intensity of light scattered from IgG solutions kept at constant temperature in the vicinity of $T_m$, in the absence and presence of polymer, are shown in Figure 5 for mixed IgG/polymer solutions containing 100 mM NaCl. To facilitate comparisons, the scattered intensity was normalized to $I_0$, the scattered light intensity of the solution of IgG alone under investigation prior to heating. The results of blank experiments carried out with a solution of IgG (0.5 mg·mL$^{-1}$) without added polymer are shown in Figure 5A. In agreement with previous results, a significant increase of the scattered light intensity occurred in IgG solutions as their temperature reached 65 °C. The initial slope of the increase in scattered light intensity measured for mixed PAA5/IgG solutions heated to 55, 60, and 65 °C was almost the same as the value obtained for solutions of IgG heated to 65 °C, confirming that this polymer does not improve the IgG stability in buffer containing 100 mM NaCl (Table 2 and Figure 5A,B).

**Table 2. Colloidal Stability Ratio, $W$ (×10$^{-6}$), Calculated from Eqs 3 and 4 and Intensity Variations in Figure 5, for IgG and IgG/Polymer Mixtures Incubated Near the Temperature of Thermal Denaturation in 100 mM NaCl, 20 mM Sodium Phosphate Buffer pH 6.8**

| $T$ (°C) | IgG | IgG/PAA5 | IgG/PAA5−3C18 | | IgG/PAA5−25C8−40C3 | |
|---|---|---|---|---|---|---|
| 55 | 240 | 200 | 660[a] | NA[b] | 110[a] | 33[b] |
| 60 | 16 | 9.8 | 2900[a] | NA[b] | 72[a] | 8.0[b] |
| 65 | 2.0 | 1.3 | 720[a] | NA[b] | 72[a] | 3.6[b] |

[a]Apparent $W$ corresponding to slopes estimated by linear extrapolation beyond 1 h incubation. [b]Values corresponding to slopes at short times (<30 min); NA: fit to eq 3 not applicable (does not extrapolate to zero at time zero).

The changes with time of the scattered light intensity recorded at 55, 60, and 65 °C for mixed IgG/PAA5−3C18 and IgG/PAA5−25C8−40C3 solutions are presented in Figure 5C,D. The increase in the scattered light intensity with incubation time was much smaller than in the case of IgG alone (note the difference in the scales of the ordinates Figure 5C,D vs Figure 5A,B). The change of intensity by a factor <10 observed in these solutions betrays a negligible contribution of strongly scattering aggregates, in agreement with the estimates of sizes derived from FCS measurements ($N_{agg}$ < 10 and possibly <2 in similar conditions). The stability of mixed IgG/PAA5−25C8−40C3 solutions seemed to decrease after several hours of incubation above 55 °C, as reflected by the slow upward drift of scattered light intensity. In mixed IgG/PAA5−3C18 solutions, the temperature-dependent increase of scattered light intensity was close to experimental error, which corroborates data from DSC and FCS on the thermal stability of IgG in these conditions. Similar conclusions can be drawn from the variation


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of apparent hydrodynamic radii (Figure S9 in SI) measured by dynamic light scattering in the same samples.

A colloid-like aggregation mechanism was used to analyze the onset of the increase with time of the scattering intensity. This model was shown previously to be valid to study the thermal denaturation of IgG.[30] It predicts that during the early stage, aggregation results from random collisions of proteins that yield a linear variation with time of the scattered intensity according to eq 3 (Smoluchowski theory on kinetics of coagulation[32]):

$$I(t) = I_0(1 + n_0 \cdot k_s \cdot t) \tag{3}$$

where $I_0$ is the initial (at $t = 0$) scattered light intensity of a monomeric IgG solution at 25 °C, $n_0$ is the IgG concentration (in number·m$^{-3}$), $k_s$ is the barrier-controlled rate constant (in m$^3$·s$^{-1}$). The slope of $(I(t) - I_0)/I_0$ gives $k_s$, which can be expressed as

$$k_s = \frac{k_s^0}{W} = \frac{4k_B T}{3\eta W} \tag{4}$$

where $k_B$ is the Boltzmann constant, $T$ is the incubation temperature, $\eta$ is the solution viscosity, and $W$ is the colloidal stability ratio.

In this framework, $k_s$ varies in proportion to the reciprocal of the colloidal stability ratio, $1/W$ (eq 4), which measures the average number of collisions between IgG molecules that are required to form one protein−protein permanent association. According to this interpretation, the chance for a "sticky" collision to occur between two IgG molecules in solutions of IgG alone or in the presence of PAA150 increases by about 10 times with each 5 °C increase of the temperature in the vicinity of $T_m$ (Table 2). The situation in the case of mixed IgG/PAA5−3C18 and IgG/PAA5−25C8−40C3 is quite different. The scattered light intensity increases markedly in the initial phase, up to an incubation time of 1 h and much slower at longer incubation times (Figure 4C,D). Also, the overall increase in scattered light intensity is weak. Reliable $W$ values could not be obtained from eq 3 and data at short time for the IgG/PAA5−3C18 mixed solutions, since the initial increase in scattered light intensity was too abrupt and did not extrapolate to zero at time zero in Figure 5C. We tentatively propose that, in the first (fast) kinetic regime, complexes form between one or several polymer chain(s) and a few proteins (or oligomers). The second regime showing slower kinetics reflects the onset of thermal aggregation. From the rate constant at long time, we estimated the values of $W$ listed in Table 2 for mixed IgG/PAA5−25C8−40C3 solutions. High $W$ values are good indicators of the efficiency of a polymer to shield the attractive interaction between thermally destabilized proteins. The apparent $W$ estimated by this method includes unavoidably the effects of both a possible change of the fraction of aggregation-prone IgG molecules as they are sequestered by polymer chains and combinations of signals due to IgG−IgG association and growth of IgG/polymer complexes. In other words, the changes in scattered light intensity cannot be unequivocally ascribed to protein−protein collisions (inter-complex collisions may also generate aggregates, for instance). Here, we use eq 4 to estimate an apparent $W$ as an index of the effectiveness of a kinetic protection, choosing somewhat arbitrarily to use in eq 3 a concentration equal to the total IgG concentration. The increase in apparent $W$ corresponds to both the decrease of free, or aggregation prone, proteins and to the higher colloidal stability of protein/polymer complexes, compared to free IgG. PAA5−3C18 differs from PAA5−25C8−40C3 in that it leads to much higher apparent $W$ values due to the nearly zero drift in scattered intensity within a few hours (e.g., at 60 °C variation are close to the experimental error) and persisting over the entire temperature range (55−65 °C; Table 2, values quoted with "*a*" exponent). The presence of PAA5−25C8−40C3 certainly slows down the aggregation rate but it does not prevent it entirely. Nonetheless, at 65 °C the presence of PAA5−25C8−40C3 decreases the aggregation rate by a factor of about 30 compared to free IgG.

**On the Practical Use of Acrylate Copolymers as Protective Agents.** The results of this investigation provide a strong indication that poly(acrylate) derivatives enhance the stability of polyclonal IgG subjected to thermal stress, a phenomenon that could be of practical use. Coulomb interactions drive the formation of soluble complexes, especially in solutions of low ionic strength, and, with additional stabilizing contribution of hydrophobic interaction, slow down significantly the thermal aggregation of IgG. Izumrudov et al. in their studies of mixed basic (cationic) enzymes and polyanions reached similar conclusions,[33,34] while Schwinte et al. reported that polyelectrolytes prevent protein aggregation at ambient temperature.[23] Our observations extend these studies to multidomain proteins of pharmaceutical interest. It is important to note that previous studies exploited electrostatic interactions between oppositely charged proteins and polymers. In the studies described here, both partners were negatively charged, albeit with IgG close to its isoelectric point, as observed by capillary electrophoresis. Coulomb interactions with PAA5 or PAA150, in solutions of low ionic strength, also protect proteins close to their isoelectric point, irrespective of the presence or not of hydrophobes on the polymer chain. Such electrostatically driven binding between polyelectrolytes and antibodies may be a general phenomenon, as suggested by a recent study of systems containing monoclonal antibodies and alginate.[35] In solutions of low ionic strength, polyanions can bind strongly to cationic patches of proteins.[36] Stabilization by polyanions may be effective as soon as the charge of proteins approaches zero and does not prevent tight association of the polymer on the cationic regions on the protein. The association of a protein with poly(acrylate) chains brings additional resistance to stress in a manner comparable to immobilization/encapsulation in colloid carriers.[37−39]

From the viewpoint of applications, complexation of IgG with polyelectrolytes has several advantages. The Coulomb interactions weaken with increasing ionic strength up to about 100 mM, which may facilitate the release of active proteins under physiological conditions. Futhermore, hydrophobic interaction can be used in addition to Coulomb association to optimize the thermal stabilization. The low amount of polymer required to achieve protection is another important advantage of the association with poly(acrylates). In comparison with osmolytes, which are only effective at molar concentrations, or with conventional detergents added at mM concentration in current formulations, a 1:1 polymer/IgG weight ratio is effective to markedly slow down aggregation. As evidenced by DSC thermograms and FCS measurements, no significant difference was observed between IgG formulations in the presence of short PAA5 or long PAA150, implying that with the longer chains, protection is reached for a 1:1 stoichiometric ratio. All these features call for the use of polyelectrolytes to stabilize therapeutic antibodies, a point that will thus deserve further studies focusing on biologically safer polymer chains.

**Physical Origin and Mechanism of Protection against Thermal Aggregation.** It is generally assumed that thermally induced protein aggregation involves partial unfolding of lesser stable domains and exposure to water of nonpolar/hydrophobic residues originally buried in the core of the protein.[40] Based on these premises, amphiphilic nonionic polymers are proposed to "cap" water-exposed nonpolar residues and shield interprotein attraction (for thermoresponsive polymers, see refs 10, 14, and 41−43, and for hydrophobic trapping in polymers, see refs 44 and 45). The fact that hydrophobic polymer/protein association exists and slows down the aggregation rate is usually inferred from indirect evidence, based on experimental correlations between polymer hydrophobicity and protection efficiency.

Results from FCS, CZE, and DLS reported here demonstrate that IgG form complexes with PAA chains, but hydrophobic binding does not emerge as the sole origin of protection. Complexation with amphiphilic or nonmodified, hydrophilic, PAA polymers is condition-dependent (effects of ionic strength and temperature) as is the resulting degree of protection achieved against aggregation. Presented in Scheme 2 is an overall view of which species were formed in the different conditions and whether stability, or slower aggregation, or no contribution of polymer occurred upon thermal stress. The section below summarizes the results that guided us to establish Scheme 2. First, DSC results indicate that at temperature below 40−45 °C, IgG is folded in the absence or presence of polymers. The fraction of (folded) IgG present under the form of complexes with polymers at low $T$ is negligible in the concentration window studied, except in the case of PAA-3C18 at low ionic strength. From this result, it appears that binding on folded IgG is not necessarily required to achieve protection. Complexation is temperature-dependent, as shown by FCS results. In 100 mM NaCl solutions, IgG associates with the amphiphilic polymers as the solution reaches temperatures near the onset of unfolding. At lower ionic strength in the absence of NaCl, the formation of complexes at the onset of IgG unfolding is also effective with the hydrophilic nonmodified PAA chains. FCS points to a low IgG/polymer chain stoichiometry of the complexes (near $T_m$, with PAA150−3C18 $N_{agg}$ is of the order of one, whereas complexes with PAA150 at low ionic strength have $N_{agg} < 6$). A simplified picture shown in Scheme 2 of one (destabilized and partially unfolded) IgG surrounded by one long polymer chain is thus representative of the state of association reached at the initial stage of the thermal stress. The colloidal stability of these complexes depends on the hydrophobicity of the polymer and of the ionic strength. An increase of the aggregation number with increasing temperature, as measured by FCS (PAA) or the slow but continuous aggregation as characterized by light scattering (PAA-25C8−40C3) point to the improvement of metastability of IgG (i.e., a decrease of the probability of IgG-IgG sticky collision). But both PAA and PAA-25C8−40C3 fail to fully prevent aggregation. Finally, PAA-3C18 polymers were the best protective agents that preserved both low $N_{agg}$ and, within experimental uncertainties, stopped the aggregation, which is indicated in Scheme 2 by the term "protection". Prevention of aggregation was not accompanied by enhanced conformational stability, as shown by the persistence of DSC peak(s) in a similar range of $T$, irrespective of the presence or not of polymers. The marked effect of polymer complexation on the enthalpy of IgG denaturation suggests in addition that association with PAA and its derivatives significantly perturbs the unfolding of IgG. In general, complexation of proteins with polyelectrolytes favors unfolding.[16,33,46−49] Future optimization of protection may therefore focus on combining the colloidal stabilization with better thermodynamic stability, for instance, by introducing kosmotropic side groups in the polymer chains.

## CONCLUSIONS

A molecular description of the temperature-dependent interactions between IgG and amphiphilic polyelectrolytes has emerged from the combined DSC, SLS, FCS, and CZE results. An overview of the IgG/polymers interactions at play in mixed systems heated from room temperature to the IgG aggregation temperature is given in Scheme 2, where we illustrate the role of PAA derivatives on IgG unfolding, association, and aggregation via Coulomb and hydrophobic interaction. Below the unfolding temperature, IgG is free in solution, except in solutions of low ionic strength containing PAA grafted with octadecyl chains where C18 chains undergo hydrophobic interactions with IgG. Colloidal stability and protection against aggregation appear to correlate with the formation of complexes near the unfolding temperature. Binding onto highly charged polymer chains presumably hinders interprotein contacts. The complexation may maintain low degree of IgG aggregation, $N_{agg}$, and even IgG monomer up to 85 °C (C18-containing polymers) or makes the aggregation to occur more gradually without preventing the formation of oligomers ($N_{agg} \approx 10$, PAA and PAA5−25C8−40C3). The weakening of the electrostatic attraction between IgG and polyelectrolytes in saline solutions (100 mM NaCl) suggests a simple route to control the release of IgG from the complexes. Another major advantage of the formulations described here is that of much less polymer is needed (1:1 w/w) compared to conventional osmolytes and detergents commonly used to stabilize protein formulations.

## ASSOCIATED CONTENT

### Supporting Information

UV−vis spectra before and after DSC, additional thermograms with short and long polymers, electropherograms at varying PAA-3C18/IgG ratios, preparation of samples for FCS, and radius of IgG/polymer, as measured from light scattering. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## REFERENCES


(1) Wang, W.; Singh, S.; Zeng, D. L.; King, K.; Nema, S. *J. Pharm. Sci.* **2007**, *96*, 1−26.
(2) Manning, M. C.; Chou, D. K.; Murphy, B. M.; Payne, R. W.; Katayama, D. S. *Pharm. Res.* **2010**, *27*, 544−575.
(3) Chen, C.; Constantinou, A.; Deonarain, M. *Expert Opin. Drug Delivery* **2011**, *8*, 1221−1236.
(4) Ryan, S. M.; Mantovani, G.; Wang, X. X.; Haddleton, D. M.; Brayden, D. J. *Expert Opin. Drug Delivery* **2008**, *5*, 371−383.
(5) Frokjaer, S.; Otzen, D. E. *Nat. Rev. Drug Discovery* **2005**, *4*, 298−306.
(6) Abbas, S. A.; Sharma, V. K.; Patapoff, T. W.; Kalonia, D. S. *Pharm. Res.* **2012**, *29*, 683−694.
(7) Ahrer, K.; Buchacher, A.; Iberer, G.; Jungbauer, A. *J. Biochem. Biophys. Methods* **2006**, *66*, 73−86.
(8) Garidel, P.; Hoffmann, C.; Blume, A. *Biophys. Chem.* **2009**, *143*, 70−78.
(9) Rudiuk, S.; Cohen-Tannoudji, L.; Huille, S.; Tribet, C. *Soft Matter* **2012**, *8*, 2651−2661.
(10) Nomura, Y.; Sasaki, Y.; Takagi, M.; Narita, T.; Aoyama, Y.; Akiyoshi, K. *Biomacromolecules* **2005**, *6*, 447−452.
(11) Yoshimoto, N.; Hashimoto, T.; Felix, M. M.; Umakoshi, H.; Kuboi, R. *Biomacromolecules* **2003**, *4*, 1530−1538.
(12) Ge, X.; Guan, Y.-X.; Chen, J.; Yao, Z.; Cao, K.; Yao, S.-J. *J. Appl. Polym. Sci.* **2009**, *114*, 1270−1277.
(13) Sedlak, E.; Fedunova, D.; Vesela, V.; Sedlakova, D.; Antalik, M. *Biomacromolecules* **2009**, *10*, 2533−2538.
(14) Liang, L.; Yao, P.; Jiang, M. *Biomacromolecules* **2006**, *7*, 1829−1835.
(15) Gao, G.; Yao, P. *J. Polym. Sci., Part A: Polym. Chem.* **2008**, *46*, 4681−4690.
(16) Stogov, S. V.; Izumrudov, V. A.; Muronetz, V. I. *Biochemistry (Moscow)* **2010**, *75*, 437−442.
(17) Xu, Y. S.; Mazzawi, M.; Chen, K. M.; Sun, L. H.; Dubin, P. L. *Biomacromolecules* **2011**, *12*, 1512−1522.
(18) Seyrek, E.; Dubin, P. L.; Tribet, C.; Gamble, E. A. *Biomacromolecules* **2003**, *4*, 273−282.
(19) Ladam, G.; Gergely, C.; Senger, B.; Decher, G.; Voegel, J. C.; Schaaf, P.; Cuisinier, F. J. G. *Biomacromolecules* **2000**, *1*, 674−687.
(20) Henzler, K.; Haupt, B.; Lauterbach, K.; Wittemann, A.; Borisov, O.; Ballauff, M. *J. Am. Chem. Soc.* **2010**, *132*, 3159−3163.
(21) Liang, L.; Yao, P.; Jiang, M. *Biomacromolecules* **2005**, *6*, 2748−2755.
(22) Wittemann, A.; Ballauff, M. *Anal. Chem.* **2004**, *76*, 2813−2819.
(23) Schwinte, P.; Ball, V.; Szalontai, B.; Haikel, Y.; Voegel, J. C.; Schaaf, P. *Biomacromolecules* **2002**, *3*, 1135−1143.
(24) Ma, D. W.; Martin, N.; Herbet, A.; Boquet, D.; Tribet, C.; Winnik, F. M. *Chem. Lett.* **2012**, *41*, 1380−1382.
(25) Gohon, Y.; Giusti, F.; Prata, C.; Charvolin, D.; Timmins, P.; Ebel, C.; Tribet, C.; Popot, J. L. *Langmuir* **2006**, *22*, 1281−1290.
(26) (a) Borrega, R.; Tribet, C.; Audebert, R. *Macromolecules* **1999**, *32*, 7798−7806. (b) J.Collet, J.; Tribet, C.; Gareil, P. *Electrophoresis* **1996**, *17*, 1202−1209.
(27) Sengupta, P.; Garai, K.; Balaji, J.; Periasamy, N.; Maiti, S. *Biophys. J.* **2003**, *84*, 1977−1984.
(28) Vermeer, A. W. P.; Norde, W. *Biophys. J.* **2000**, *78*, 394−404.
(29) Vermeer, A. W. P.; Bremer, M.; Norde, W. *Biochim. Biophys. Acta, Gen. Subj.* **1998**, *1425*, 1−12.
(30) Andersen, C. B.; Manno, M.; Rischel, C.; Thorolfsson, M.; Martorana, V. *Protein Sci.* **2010**, *19*, 279−290.
(31) Pouliquen, G.; Tribet, C. *Macromolecules* **2006**, *39*, 373−383.
(32) Hunter, R. J. *Foundations of Colloid Science*; Oxford University Press: New York, 2001; pp 616−627.
(33) Ivinova, O. N.; Izumrudov, V. A.; Muronetz, V. I.; Galaev, I. Y.; Mattiasson, B. *Macromol. Biosci.* **2003**, *3*, 210−215.
(34) Shalova, I. N.; Asryants, R. A.; Sholukh, M. V.; Saso, L.; Kurganov, B. I.; Muronetz, V. I.; Izumrudov, V. A. *Macromol. Biosci.* **2005**, *5*, 1184−1192.
(35) Schweizer, D.; Schonhammer, K.; Jahn, M.; Gopferich, A. *Biomacromolecules* **2013**, *14*, 75−83.
(36) Cooper, C. L.; Dubin, P. L.; Kayitmazer, A. B.; Turksen, S. *Curr. Opin. Colloid Interface Sci.* **2005**, *10*, 52−78.
(37) Yan, M.; Liu, Z. X.; Lu, D. N.; Liu, Z. *Biomacromolecules* **2007**, *8*, 560−565.
(38) Hirakura, T.; Yasugi, K.; Nemoto, T.; Sato, M.; Shimoboji, T.; Aso, Y.; Morimoto, N.; Akiyoshi, K. *J. Controlled Release* **2010**, *142*, 483−489.
(39) Basset, C.; Harder, C.; Vidaud, C.; Dejugnat, C. *Biomacromolecules* **2010**, *11*, 806−814.
(40) Dill, K. A.; Shortle, D. *Annu. Rev. Biochem.* **1991**, *60*, 795−825.
(41) Cavalieri, F.; Chiessi, E.; Paradossi, G. *Soft Matter* **2007**, *3*, 718−724.
(42) Lu, D.; Wu, J.; Liu, Z. *J. Phys. Chem. B* **2007**, *111*, 12303−12309.
(43) Kamiyama, T.; Sadahide, Y.; Nogusa, Y.; Gekko, K. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1999**, *1434*, 44−57.
(44) Morimoto, N.; Endo, T.; Iwasaki, Y.; Akiyoshi, K. *Biomacromolecules* **2005**, *6*, 1829−1834.
(45) Constancis, A.; Meyrueix, R.; Bryson, N.; Huille, S.; Grosselin, J. M.; Gulik-Krzywicki, T.; Soula, G. *J. Colloid Interface Sci.* **1999**, *217*, 357−368.
(46) Wittemann, A.; Ballauff, M. *Macromol. Biosci.* **2005**, *5*, 13−20.
(47) Khondee, S.; Olsen, C. M.; Zeng, Y. H.; Middaugh, C. R.; Berkland, C. *Biomacromolecules* **2011**, *12*, 3880−3894.
(48) Mazzaferro, L.; Breccia, J. D.; Andersson, M. M.; Hitzmann, B.; Hatti-Kaul, R. *Intl. J. Biol. Macromol.* **2010**, *47*, 15−20.
(49) Sun, J.; Ruchmann, J.; Pallier, A.; Jullien, L.; Desmadril, M.; Tribet, C. *Biomacromolecules* **2012**, *13*, 3736−3746.