Charge and Size Drive Spontaneous Self-Assembly of Oppositely Charged Globular Proteins into Microspheres

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Controlled interactions and assembly of proteins with one another promise to be a powerful approach for generating novel supramolecular architectures. In this study, we report on the ability of oppositely charged globular proteins to self-assemble into well-defined micrometer-sized spherical particles under specific physicochemical conditions. We show that microspheres were spontaneously formed in all binary protein mixtures tested once the physicochemical conditions were optimized. The optimal pH value, initial protein concentrations needed to form microspheres, and protein stoichiometry in these microspheres varied and depended on the structural features of the mixed proteins. We show that charge compensation is required but not sufficient to guide optimal protein assembly and organization into microspheres. Size difference between protein couples (acidic and basic) is a key element that defines optimal pH value for microsphere formation and the protein molar ratio in the formed microspheres. Our findings give new elements that can help to predict the assembly behavior of various proteins in mixed systems.

Introduction

Controlled self-assembly of biomacromolecules has recently attracted considerable attention because this process leads to new materials with potential applications in various fields such as nano-, medical, or food technologies. Molecular self-assembly is a process during which molecules placed under specific conditions spontaneously form ordered supramolecular structures through noncovalent binding.[1] Proteins were reported to be ideal biomaterials to obtain tailor-made new nanoparticles with various architectures.[2] Hence, throughout adequate control of their self-assembly process, a large diversity of supramolecular structures including aggregates, fibres, nanotubes or spherulites can be generated from globular proteins.

Presently, only few studies describe the spontaneous protein−protein interactions and assembly into nano- or micrometer sized objects constituted of at least two different proteins. Natural products are complex systems with various globular proteins that could associate to generate supramolecular structures in which weak noncovalent attractive interactions constitute the main driving force.[3] Howell et al. first reported the predominant formation of an insoluble precipitate after mixing lysozyme with whey proteins β-lactoglobulin or α-lactalbumin.[4] Biesheuvel et al. formed supramolecular structures throughout interaction of native lysozyme and its chemically modified oppositely charged form (succinylated lysozyme).[5] Recently, we evidenced that calcium-depleted apo-α-lactalbumin (ALA) and hen egg white lysozyme (LYS) interact and self-assemble to form different supramolecular structures in a temperature-dependent manner. Amorphous aggregates were observed at 5 °C while well-defined microspheres were obtained at 45 °C.[6] To our knowledge, this is the first example of the assembly of protein binary mixtures into well-defined microspheres. The aim of the present study was to determine whether the formation of a spherical particle could be a generic form of aggregation using well-known model proteins, i.e., LYS, ovalbumin (OVA), avidin (AVI), and bovine serum albumin (BSA). Our objective is to gain more knowledge on the physicochemical parameters that favor protein−protein interactions and self-assembly into spherical supramolecular structures.

OVA, LYS, AVI, and BSA are four globular proteins that differ in both molecular mass and isoelectric point (pI). OVA is a phosphoglycoprotein with 385 amino acid residues and a molecular mass of about 45 500 $g \cdot mol^{-1}$. It has one internal disulfide bond and four free sulphydryl groups. Half of its amino acid residues are hydrophobic, and a third are charged, mainly acidic residues, giving the protein a pI of 4.8.[7] LYS, a well-known enzyme, has 129 amino acid residues and a molecular mass of 14 305 g mol^{-1}. Its three-dimensional structure is highly stabilized by four disulfide bonds. It has a strong basic character with a pI of 10.7.[8] AVI is a basic (pI of 9.5[9]) non covalent homotetrameric glycoprotein. The monomers contain 128 amino acid residues, giving the protein an overall molecular mass of about 67 000 $g \cdot mol^{-1}$. Although each monomer shares interactions with the three others, AVI is usually regarded as a dimer of dimers. The dimers are constituted of monomers highly stabilized by a multitude of polar and hydrophobic interactions, and the interaction between the two dimers was reported to be weaker and mainly hydrophobic.[10] BSA is an acidic protein (pI near 5.0[11−13]) that contains 583 amino acid residues, leading to a molecular mass of about 66 500 $g \cdot mol^{-1}$. It has 17 disulfide bonds and one free sulphydryl group and shares 76% identity with human serum albumin.

2. Experimental Methods

2.1. Materials. LYS hydrochloride and AVI were from Ovonor (Ovonor SA, Annezin les Bethunes, France). LYS hydrochloride contained 95% LYS and 3% chloride ions. OVA was purified as previously described,[14] and the purity was 95%, as determined by reverse-phase high-performance liquid chro-

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matography (HPLC). BSA (purity >98%), fluorescein isothiocyanate (FITC) and rhodamine B isothiocyanate (RITC) were from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France). The salt content of protein powders is negligible and does not significantly modify the overall ionic strength of the solutions used.

2.2. Turbidity Measurements. Turbidity measurements were performed using a Jenway 6505 UV−vis spectrophotometer (Jenway, Chelles, France). Absorbance was read at 600 nm and converted into a turbidity (τ) value using the following relationships: $\tau = (2.303A_{600\ nm})/l$, where $A_{600\ nm}$ is the absorbance at 600 nm and l is the light path length in the cell (1 cm). Turbidity was measured 5 min after mixing the two proteins. When the effect of pH was investigated, protein mixtures were prepared in 10 mM Tris-HCl buffer, 10 mM Bis-Tris buffer, or 10 mM ethanolamine buffer. The ionic strength was adjusted to 10 mM. The protein concentrations were 170 μM LYS and 60 μM OVA for LYS/OVA mixtures, 3.0 μM AVI and 4.4 μM OVA for AVI/OVA mixtures, and 58.5 μM and 12.5 μM for LYS/BSA mixtures. To study the effect of ionic strength on protein assembly, LYS and OVA samples were prepared in 10 mM Tris-HCl buffer, pH 8.0, with ionic strength varying from 6 to 20 mM. In the case of AVI/OVA mixtures, turbidity was measured in 10 mM Bis-Tris buffer, pH 6.8, with ionic strength varying from 3 to 25 mM. In the case of LYS/BSA mixtures, turbidity was measured in 10 mM ethanolamine buffer, pH 8.9, with ionic strength varying from 8.5 to 25 mM. The protein concentrations were the same as above.

2.3. Protein Quantification by Liquid Chromatography. Protein separation and quantification were performed by cation exchange chromatography in the case of LYS/OVA and AVI/OVA mixtures or size exclusion chromatography for LYS/BSA. Protein samples were prepared by mixing OVA and LYS in 10 mM Tris-HCl buffer, pH 7.4, 8.0, and 8.6; OVA and AVI in 10 mM Bis-Tris buffer, pH 6.4 and 6.8, or in 10 mM Tris-Cl buffer, pH 8.0; and LYS and BSA in 10 mM Tris-HCl buffer, pH 7.4, or in 10 mM ethanolamine buffer, pH 8.9 and 10.0, in a final volume of 200 μL. Ionic strength was kept constant at 10 mM. LYS, OVA, and AVI concentrations were 210, 44, and 46 μM, respectively in the cases of LYS/OVA and AVI/OVA systems. For the LYS/BSA system, LYS and BSA concentrations were 175 and 37.5 μM, respectively. The mixtures were kept at room temperature for at least 18 h (time needed for the spheres to coalesce and sediment). Protein quantifications were performed in supernatants diluted five times in adequate chromatographic buffer.

Cation exchange chromatography was performed using a Biosepra S-HYPERD 10 column connected to a Waters 2695 HPLC. Proteins were eluted using a gradient from 100% buffer A (20 mM sodium acetate buffer pH 4.0) to 100% buffer B (20 mM sodium acetate buffer pH 4.0 containing 1 M NaCl) in 24 min at a flow rate of 1 mL·min^{-1}.

Size exclusion chromatography was performed using a Biosepra S2000 column (300 × 7.80 mm) equilibrated in 20 mM acetate buffer, pH 5.0, 0.15 M NaCl, and 0.5% sodium dodecyl sulfate (running buffer). Protein samples were eluted at a constant flow rate of 1 mL·min^{-1} with the same buffer for 20 min. In both cases, absorbance was monitored at 214 and 280 nm simultaneously using a Waters 2487 Dual Absorbance detector.

2.4. Protein Labeling. Proteins were labeled separately using covalently linked fluorescent dyes FITC or RITC. LYS, OVA, and AVI were dissolved in 100 mM phosphate buffer pH 8.0 at concentrations of 350, 117, and 76 μM, respectively. Then, the dyes dissolved in ultrapure ethanol at 5 mg·mL^{-1} were added dropwise to the protein solution until a final protein/dye molar ratio of 0.5 was reached. Subsequently, the samples were kept in the dark, at room temperature for 1 h. Free dyes were eliminated by dialysis (nominal cutoff of 6−80000 Da) against 10 mM Tris-HCl buffer, pH 7.4, containing 0.6 M NaCl. Salts were then removed by dialysis against Milli-Q water before filtering through a 0.2 μm membrane and freeze-drying. Whatever the protein, the labeling rate does not exceeded 5% of labeled protein molecules as assessed by mass spectrometry analyses (results not shown).

TABLE 1: pH Dependence of OVA, LYS, and AVI Net Charges[a]

pH	Z_{OVA}	Z_{LYS}	Z_{AVI}	Z_{BSA}
3	+28.0	+13.0	+43.1	
4	+12.0	+10.5	+31.4	
5	−1.0	+9.0	+22.9	
5.5		+8.5		
6	−8.0	+8.0	+20.1	−4.0
7	−12.0	+7.5	+18.7	−7 to −10.0
8	−14.0	+7.0	+16.5	−12.5 to −15.0
9	−16.0	+6.0	+13.5	−15.0 to −22.0
10	−20.0	+3.5	−2.7	
11	−28.0	0.0	−19.11	
MW (g mol^{-1})	45500	14305	4 × 14343	66500

[a] Experimental values for OVA and LYS are those reported by Teske et al.[17] The values for BSA are given in the pH range covered in this study.[12,13,18] Values for AVI tetramer, constructed using PDB file 1VYO,[38] were calculated using PROPKA v2.0.[15,16] Molecular weights of proteins are also indicated.

2.5. Optical Microscopy. Phase contrast micrographs were obtained using an Olympus BX51TF microscope equipped with an Olympus DP11 camera at a magnification of 100×. Confocal scanning laser microscopy was performed on an inverted TE2000-E microscope (Nikon, Champigny-sur-Marne, France) equipped for differential interference contrast (DIC). Argon ion and helium/neon lasers emitting at 488 and 543 nm, respectively, were used for fluorescence microscopy.

3. Results

Four model proteins with different physicochemical properties were used to assess the ability of globular proteins to assemble into spherical particles. Table 1 shows the molecular masses of the chosen proteins and their electric charges at various pH. The net charge of an AVI tetramer was calculated using PROPKA v2.0,[15,16] while the values indicated for LYS, BSA, and OVA were obtained experimentally.[12,13,17,18] Between pH 5.0 and about 10.0, LYS and AVI are positively charged, while OVA and BSA are negatively charged.

3.1. OVA/LYS Assembly. The formation of supramolecular structures was monitored by turbidity measurements in LYS/OVA mixtures at concentrations of 170 and 60 μM, respectively. Immediately after mixing, turbidity increased at room temperature, to reach a plateau after 5 min. On the basis of turbidity measurements, it was assured that interactions between the two proteins led to supramolecular structures from pH 7.0 to pH 8.6 (Figure 1A). The maximal value of turbidity was reached at pH 8.0. Moreover, optical microscopy revealed that the supramolecular structures formed were spherical from pH 7.4 to pH 8.6 (Figure 1A). The spheres were heterogeneous in size, with diameter between the lower limit of optical microscopy (250−300 nm) and 8−10 μm at pH 8.0. From pH 6.8 to 7.4, turbidity increased, but no spheres could be observed using optical microscopy, suggesting that the formed objects are probably too small to be detected. At pH value higher than 8.0,

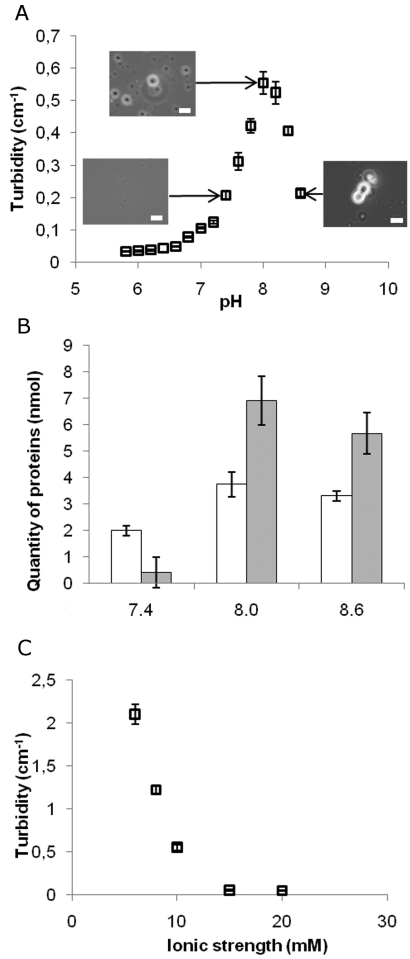

Figure 1. Formation of microspheres between OVA and LYS at room temperature. (A) Supramolecular assembly in OVA (44 μM) and LYS (170 μM) mixtures at various pH evidenced by turbidity measurements and optical microscopy observations (scale bars = 5 μm). (B) Quantities of OVA (white bars) and LYS (gray bars) found in formed microspheres at different pH and fixed ionic strength of 10 mM. (C) Effect of ionic strength on microsphere formation at optimal pH (pH 8.0) as determined by turbidity measurements.

some microspheres fused rapidly to form larger aggregates with undefined shapes.

To determine the quantity of proteins involved in the microspheres, suspensions of particles were prepared by mixing LYS (210 μM) and OVA (44 μM) in 10 mM Tris-HCl buffer, pH 7.4, 8.0, and 8.6, at a constant ionic strength of 10 mM. Figure 1B confirms that the highest amount of protein included in the microsphere fraction was observed at pH 8.0. In these conditions, 42.6 $\pm$ 5.0% of OVA were incorporated in the microspheres as determined by cation exchange chromatography. Moreover, the LYS/OVA ratio in the microspheres was 1.85 $\pm$ 0.10. Considering the data presented in Table 1, the LYS/OVA molar ratio in the spheres then suggests that the net charge of each sphere is nearly equal to 0 since the net charges of LYS and OVA are +7 and −14, respectively. At pH 8.6, the LYS/OVA molar ratio was 1.72 $\pm$ 0.31, indicating that the relative protein composition of the microspheres was not significantly modified from pH 8.0 to 8.6. On the other hand, at pH 7.4 the molar ratio decreased significantly to 0.22 $\pm$ 0.30. Taken together with the values presented in Table 1, this result indicates that the supramolecular structures formed in these conditions have an excess of negative charges. However, the structures observed in these conditions were hardly detectable, and the spherical shape could be confirmed only for the biggest ones. The smaller ones might have a distinct morphology, nonequivalent to the system observed at pH 8.0. Surprisingly, the quantity of proteins in the microspheres was very low at pH 7.4, compared to that at pH 8.6, while the turbidity values were nearly the same at these both pH values. This is confirmed by optical microscopy: at pH 7.4, only few spheres were observed, while at pH 8.6 the supramolecular structures formed are similar to those observed at pH 8.0. Therefore, at pH 7.4 the major species responsible for the increase in turbidity are small objects with undefined shape. In all experimental conditions, no supramolecular structures were detected in control samples, i.e., in pure LYS or OVA solutions.

The sensitivity of OVA (60 μM) and LYS (170 μM) assembly to ionic strength was determined in 10 mM Tris-HCl buffer pH 8.0 containing NaCl to reach final ionic strengths ranging from 6 to 25 mM. The samples were kept 5 min at room temperature before the turbidity was quantified. Ionic strength had a great influence on microsphere formation: the lower the ionic strength, the higher the rate of aggregation (Figure 1C). As soon as ionic strength reached 15 mM, no turbidity was measured.

Protein localization in formed microspheres was determined using confocal microscopy and fluorescent labeled proteins. FITC-labeled LYS (FITC-LYS) and RITC-labeled OVA (RITC-OVA) were used to prepare microspheres in 10 mM Tris-HCl buffer pH 8.0 (μ = 6 mM). Labeling did not alter the assembly process, and both proteins were shown to be homogeneously distributed into the spheres (Figure 2A). Moreover, merging the images and representing relative fluorescence intensity of each probe along one sphere diameter indicated that OVA and LYS were colocalized in regards of confocal resolution (Figure 2B), as was the case for LYS/ALA microspheres.[19]

3.2. OVA/AVI Assembly. LYS was substituted with AVI to check the ability of other basic proteins to form microspheres. The experiments were performed with AVI and OVA at concentrations of 3 and 4.4 μM, respectively. These values were lower than those for LYS/OVA mixtures because, at higher concentrations, the self-assembly process was too fast, the turbidity value was too high, and sedimentation of the formed objects occurred readily. The occurrence of an assembly process at room temperature from pH 6.2 to pH 8.2 was assessed by turbidity; the maximum value was observed at pH 6.8 (Figure 3A). Optical microscopy observations indicated that the supramolecular structures formed were spherical in the range of pH 6.4 to 8.0 (Figure 3A). The size of the spheres varied from the lower detection limit of optical microscopy to 5−10 μm.

The highest proportion of both proteins recovered in microspheres, i.e., 77.7 $\pm$ 0.3%, was obtained at pH 6.8, while slightly lower values were obtained at pH 6.4 and 8.0, i.e., 70.4 $\pm$ 0.1% and 71.5 $\pm$ 2.5%, respectively. As determined by cation exchange chromatography, the AVI/OVA molar ratios in the microspheres were 0.96 $\pm$ 0.04, 0.93 $\pm$ 0.03, and 1.05 $\pm$ 0.06 at pH 6.4, 6.8, and 8.0, respectively. These protein ratios could not be explained in terms of electroneutrality since, in the studied pH range, the charge of OVA changes significantly (Table 1).

As for the LYS/OVA system, the two proteins were also found to be colocalized in the formed spheres at pH 8.0. AVI (3 μM) and OVA (4.4 μM) assembly was also found to be highly sensitive to ionic strength, as shown by turbidity measurements (Figure 3C). While the assembly process between the two proteins was favored at low ionic strength, no supramolecular structures were detected above an ionic strength value of 15 mM.

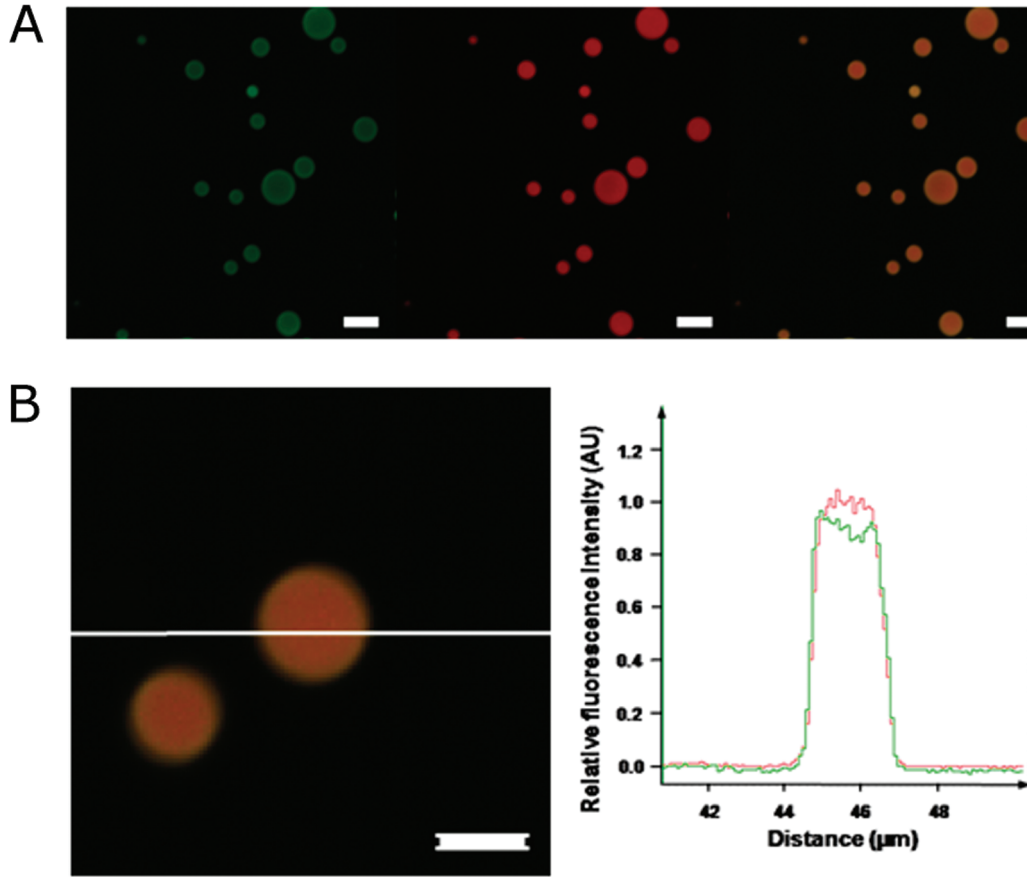

Figure 2. Confocal laser scanning microscopy observations of FITC-LYS and RITC-OVA. (A) Dyes were excited separately (green and red spheres, respectively) or simultaneously (orange spheres). (B) Relative fluorescence intensity of FITC (green line) and RITC (red line) were measured along the diameter of one sphere. Scale bars = 5 μm (A) and 2 μm (B).

3.3. LYS/BSA Assembly. The protein molar ratio in the microspheres was shown to be specific for each system. This was further studied using BSA, a large acidic protein, in mixture with LYS. Turbidity measurements of LYS/BSA mixtures indicate that supramolecular assembly occurred in the pH range of 7.4 to 10.5 (Figure 4A). The maximal value was observed at pH 8.9. From pH 7.4 to 9.2, only microspheres were observed by optical microscopy (Figure 4A). However, for higher pH values, aggregates of undefined shapes were also observed. They could result from the lower solubility of LYS since amorphous aggregation occurred in samples containing only the basic protein.[20] The size of the spheres determined by optical microscopy was less than 2 μm.

The amount of both proteins involved in microspheres as determined by size exclusion chromatography varied as a function of pH value (Figure 4B). At pH 7.4, the protein quantities included in the microspheres were too low to be detected by size exclusion chromatography. At pH 10.0, the presence of amorphous aggregates prevented correct quantification of LYS involved in microspheres (Figure 4B). At pH 8.9, where only spheres were observed, the proportion of LYS and BSA included in the microspheres was 18.1 $\pm$ 0.1% and 28.1 $\pm$ 2.3%, respectively (Figure 4B). The LYS over BSA molar ratio at this pH value was 3.1 $\pm$ 0.3. These results are in favor of electroneutrality in the microspheres (Table 1). Indeed, the net charge of LYS and BSA at pH 8.9 is +6.0 and between −15 and −22,[12,13,18] respectively. The variations of BSA charge arise from different experimental conditions used for measurements.[18] As for the two other systems, the LYS/BSA microsphere assembly is highly sensitive to ionic strength (Figure 4C). No particles were detected above an ionic strength of 20 mM.

4. Discussion

Over the last two decades, extensive research has been performed on charge-driven complex formation between two oppositely charged macromolecules, particularly in heteromolecular systems. On one hand, well-studied objects are the complex coacervate formed with proteins and polysaccharides or other polyelectrolytes with uniformly distributed and highly controlled number of charges. In these studies, objects with controlled size and various shapes were generally achieved, even if the parameters that govern the mechanism of assembly are not fully understood. On the other hand, few studies are dedicated to systems containing a mix of oppositely charged proteins.

We recently demonstrated that, under specific conditions (temperature, protein conformational state), the apo form of α-lactalbumin (ALA) and LYS interact and self-assemble into well-defined spherical particles.[6] However, the calcium-loaded-α-lactalbumin was considered to be unable to form micrometer-sized spherical particles in the presence of LYS.[6] A question then arises: is there a specific interaction between LYS and ALA that favors the formation of microspheres? In other words, would this kind of assembly be observed only for a mixture consisting of these two polypeptides? We rather assumed that the generation of microspheres from oppositely charged protein mixtures is a general assembly process, given that adequate physico-

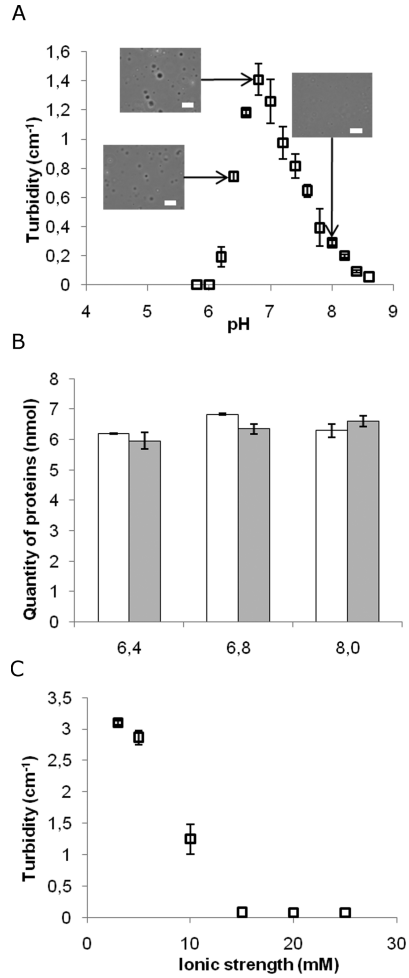

Figure 3. Formation of microspheres between OVA and AVI at room temperature. (A) Supramolecular assembly in OVA (3 μM) and AVI (4.4 μM) mixtures at various pH evidenced by turbidity measurements and optical microscopy observations (scale bars = 5 μm). (B) Quantities of OVA (white bars) and AVI (gray bars) found in the microspheres at different pH and 10 mM ionic strength. (C) Effect of ionic strength on microsphere formation at optimal pH (pH 6.8) as determined by turbidity measurements.

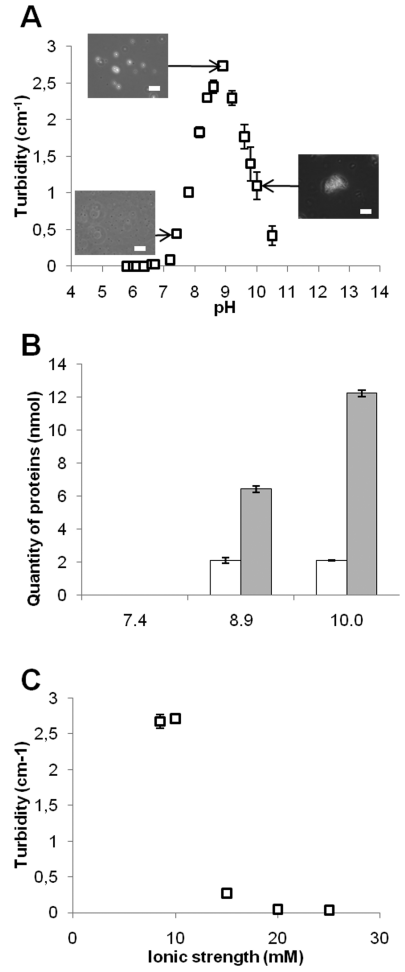

Figure 4. Formation of microspheres between LYS and BSA at room temperature. (A) Supramolecular assembly in LYS (58 μM) and BSA (12.5 μM) mixtures at various pH evidenced by turbidity measurements and optical microscopy observations (scale bars = 5 μm). (B) Quantities of BSA (white bars) and LYS (gray bars) found in the microspheres at different pH and 10 mM ionic strength. (C) Effect of ionic strength on microsphere formation at optimal pH (pH 6.8) as determined by turbidity measurements.

chemical conditions are found for each protein system. In the three binary systems studied here, we showed that they exhibit two common general trends: (i) spontaneous formation of spheres in micrometer range at room temperature and (ii) high sensitivity of formed microspheres to ionic strength above 20 mM. We discuss here the conditions favoring the formation of these spherical supramolecular assemblies in the light of the results presented here and those obtained previously with LYS/ALA.

4.1. Electrostatic Interactions Drive Protein Assembly into Spherical Supramolecular Structures. From all the systems studied, in a pH range where acidic and basic proteins carry opposite net charges, spherical particles could be observed. Moreover, the two proteins were distributed homogeneously in the spheres. As for the LYS/ALA mixture, the three systems reported here exhibit an optimum pH value for microsphere formation where charge compensation occurs. This point is confirmed at least for three of the four systems for which experimental data on net charge of proteins as a function of pH are available. Charge compensation favors growth of aggregated structures in every direction, leading to spherical structures as predicted from calculations.[21] Another explanation could be that assemblies are controlled by the existence of surface tension at the interface between the solution and the condensed phase. Sphere formation leads to surface energy gain throughout the minimization of the particle surface.[22]

Spherical particles were observed only over one pH unit range, where charge compensation probably occurs. When the pH was far from this value, no microspheres or amorphous aggregates were detected. The charge of some functional groups may not be favorable for association in micrometric spherical particles. However, the ionization state of functional groups is difficult to predict since protein−protein interactions lead to a change in the overall charge of proteins.[23] For AVI/OVA, electroneutrality of the microspheres could not be verified experimentally because the experimental titration curve of the protein is unknown. However, since the AVI/OVA ratio in the spheres is 1 and assuming that charge compensation occurs in the spheres, we assume that the net charge of the tetrameric AVI at pH 6.8 is equal to the charge of OVA, but opposite in sign, i.e., about +11. Since this value is quite different and lower than the theoretical one (Table 1), it is assumed that less positive charges than predicted are exposed on the surface of the tetrameric structure of AVI.

In all protein binary systems, the amount of particles decreased when the ionic strength of the buffer increased.

Throughout surface charge screening, the assembly process was abolished above an ionic strength of 15−20 mM. This threshold value is significantly lower than that found in the case of LYS/ALA,[24] underlying the specificity of each binary system. Indeed, microspheres of LYS/ALA were formed even in buffer with ionic strength of 54 mM,[24] a value higher than the one required to prevent assemblies in the binary systems described in the present study. This could be attributed to the molten globule state of ALA that have a more loose and flexible structure and exhibits a higher surface hydrophobicity that could allow the establishment of nonelectrostatic interactions in addition to electrostatic ones. It was reported, for instance, that, at neutral pH and ionic strength below 100 mM (low ionic strength), calcium-free ALA adopts a molten globule state.[25] Consequently, in contrast to the systems reported here, the interaction and self-assembly of ALA with LYS above 15−20 mM could be promoted throughout higher protein flexibility and surface hydrophobicity induced by Ca^{2+} removal from ALA. Whatever the binary system, the observed effect of NaCl suggests that, at least for the first steps of association, electrostatic interactions are essential to obtain microspheres.

Association and aggregation were reported for charged polyelectrolytes even at higher ionic strength.[26] For charged polyelectrolytes, micelles are observed at ionic strengths up to 500 mM.[27] One can assume that surface charge density of proteins may be much lower than for polyelectrolytes, and then any addition of salt dramatically decreases electrostatic attractions, inhibiting microsphere formation.[28] Laugel et al. showed that, at pH = $1/2(pK_{cation} + pK_{anion})$ and at an ionic strength below 5 mM, two weakly charged polyelectrolytes act as fully charged polyelectrolytes.[29] Strikingly, the pH value where microspheres carry no net charge is close to halfway between the pI's of the proteins involved in such particles for most of the systems. Consequently, a correlation exists between optimal pH for microsphere formation and average pI's of the two proteins. The most favorable pH values for sphere formation are 8.0, 7.5, and 6.8 for LYS/OVA, LYS/ALA, and AVI/OVA, respectively, and half of the sum of their pI's are 7.7, 7.4, and 7.1. For LYS/BSA mixture, the observed optimal pH of 8.9 is slightly away from the average value of the pI's, probably because of the diversity of available pI values for BSA. Even if the situation is slightly different in this last case, this correlation constitutes a good starting point to predict the behavior of other couples of globular proteins, Hence, protein−protein charge compensation and electroneutrality of the microspheres seems to be a general trend of these assemblies, reinforcing the idea that electrostatic interactions are crucial. Such electroneutrality of microspheres may explain the coalescence phenomena between individual microspheres observed for all systems after prolonged incubation time as already reported for the LYS/ALA system.[6]

4.2. Relative Molecular Size Dictates the Protein Stoichiometry in the Microspheres As Well As the Optimal pH for Their Formation. Charge compensation theory is generally used to explain the assembly between oppositely charged polymers and the stoichiometry in formed supramolecular structures. This theory is easily applicable to explain our studies in binary systems where the 1:1 stoichiometry was found at optimal pH conditions, e.g., LYS/ALA[6] and OVA/AVI. However, the results obtained with LYS/OVA and LYS/BSA systems underline the fact that charge compensation may not be the only prerequisite for optimal self-assembly of proteins into microspheres. For these two systems, the optimal pH was found to be far from the pH value where the proteins carry equivalent net charges with opposite sign, i.e., pH 6 and near 7 for LYS/OVA and LYS/BSA, respectively. The pH value for optimal assembly of LYS/OVA corresponds to the value where charge compensation was achieved with 2:1 stoichiometry. At the same time, the optimal assembly between LYS and OVA was reached at pH where 3:1 stoichiometry is required for charge compensation. In both systems, the protein in excess in the formed microspheres is the one with the smallest size. Taken together, these results underline the fact that charge compensation is not always sufficient to lead to optimal assembly between oppositely charged proteins into microspheres. We suggest that adequate conditions for optimal assembly into microspheres for each binary system correspond to those where combination of charge compensation and "size compensation" between partners occur. In other words and behind charge compensation, protein stoichiometry in assembled structures is related to their molecular size differences. To better visualize such differences, the structures of LYS, ALA, OVA, AVI, and human serum albumin are shown in Figure 5. When the overall size of negatively and positively charged molecules are of same order of magnitude, "size compensation" occurs at 1:1 stoichiometry. This is the case for LYS/ALA and AVI/OVA mixtures. LYS and ALA exhibit similar three-dimensional structures with similar dimensions of 4.5 × 3.0 × 3.0 nm^3.[30] The sizes of AVI and OVA are close to each other with dimensions of 6.0 × 4.0 × 5.5 nm^3 and 7.0 × 4.5 × 5.0 nm^3, respectively.[31,32] For the LYS/BSA system, the net charge of BSA is about 3 times higher than the net charge of one LYS molecule at optimal pH.[12,13,18,33] The dimensions of BSA are those of an equilateral triangle with 8.0 nm sides and 3 nm depth,[34] i.e., about 3 times the volume of LYS, explaining the 3-fold excess of LYS over BSA in microspheres. By the same way, the difference between calculated dimensions for LYS and OVA explain the 2:1 stoichiometry found in microspheres under optimal pH values for assembly. The same reasoning could be applied assuming the calculated surface area of the used globular proteins according to Miller et al.[35] LYS and ALA exhibit the same calculated surface area of about 58 nm^2 against 147, 180, and 275 nm^2 for OVA, AVI, and BSA, respectively. However, the use of surface area as a dimension to explain the recovered stoichiometry in microspheres results in values less than those of protein volumes, particularly for the LYS/BSA system, probably because the calculated accessible surface areas are overestimated.

The formation of specific oligomers as an initial step for the assembly process of proteins and oligopeptides has already been proposed for other aggregation mechanisms.[36] We previously

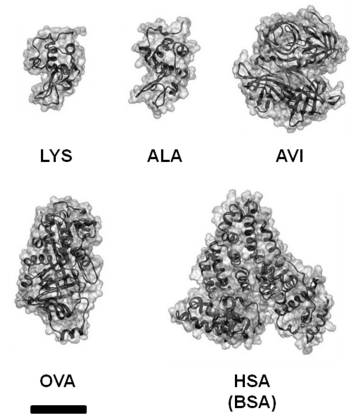

Figure 5. Representation of the structures of lLYS (PDB code: 1LYZ),[39] ALA (PDB Code: 1F6S),[40] AVI (PDB code: 1VYO),[38] OVA (PDB code: 1OVA),[31] and human serum albumin (HSA, PDB code: 1AO6).[41] HSA is presented in place of BSA because this protein lacks three-dimensional structure information. Scale bar = 3 nm.

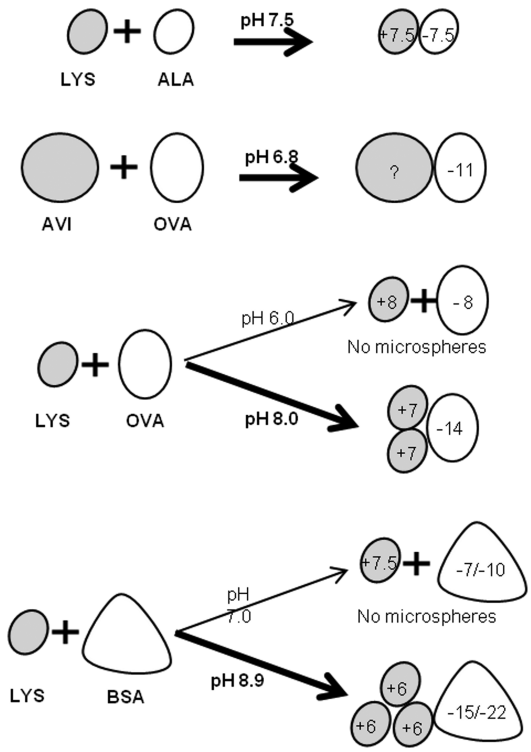

Figure 6. Schematic representation of the initial building blocks as a function of protein binary system required for optimal self-assembly into microspheres.

proposed a heterodimer as the building block for further supramolecular assembly into microspheres in the case of the LYS/ALA mixture.[37] From the present results, we suggest that optimal assembly into microspheres occurs under conditions simultaneously favoring charge as well as size compensation. Figure 6 illustrates such conditions for each studied binary system. Consequently, the number of total molecules in the building blocks that initiate self-assembly into microspheres is assumed to be system specific: heterodimers for ALA/LYS and AVI/OVA, trimer for LYS/OVA in a 2:1 molar ratio, and tetramer for LYS/BSA in a 3:1 molar ratio. When the two molecules exhibit similar size, optimal charge compensation occurs at 1:1 stoichiometry. When the size of one protein is bigger than the other, higher stoichiometry is needed for optimal charge compensation.

5. Conclusions

The present study focused on the ability of oppositely charged globular proteins to interact and self-assemble into original spherical structures in different protein binary systems. From our results, we suggest that self-assembly into microspheres is a universal form of molecular assembly as far as optimal physicochemical conditions are defined. The three binary systems studied here exhibited common properties such as high sensitivity to ionic strength and the ability to form microspheres at room temperature. However, the optimal pH conditions for microspheres formation were found to be specific for each binary system. We suggest that this specificity is linked to both charge and size compensations that lead to initial oligomers with an overall charge near zero. Hence, the difference between the sizes of the two protein partners dictates the final stoichiometry in the assembled structures.

Microspherical particles were also obtained in a mixture containing more than two proteins, e.g., OVA, LYS, and AVI (data not shown). The three proteins were also perfectly colocalized into formed microspheres. Studies are in progress to check whether protein stoichiometry in formed spheres is also linked to charge and size compensations.

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