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ORIGINAL CONTRIBUTION

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Formation of gliadin nanoparticles: Influence of the solubility parameter of the protein solvent

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Abstract Nanoparticles are produced from gliadin, a wheat gluten fraction, by precipitation in a non-solvent. In order to control their size, the solvent effect is studied by solubilizing the protein in different mixtures whose solubility parameters are close or equal to the gliadin parameter, δ_G. The determination of δ_G is performed by turbidity. The size of the nanoparticles is evaluated by QELS (quasi-elastic light scattering). The study of the size as a function of the solubility parameter of the protein solvent allows the determination of the optimal conditions for nanoparticle formation.

Key words Nanoparticles – gliadin – solubility parameter – desolvatation method

Introduction

Nanoparticles obtained from natural or synthetic polymers are currently attracting much interest as specific carriers in pharmacy, in cosmetology, in the agro-chemical industry and in other industrial fields [1–3]. For biological applications, vegetal particles have been derived from proteins [4, 5], such as gliadin extracted from gluten of wheat and vicillin or legumin extracted from pea seeds. Their potential appears to be large, especially in the targeting of active principles.

The formation of gliadin nanoparticles by desolvatation method in a non-solvent mixture, as well as their loading and controlled release capacity of all trans-retinoic acid have recently been shown [5]. It is concluded that the coacervate formation is very sensitive to environmental parameters and to the structural characteristics of the protein.

In this paper, we propose to understand the influence of the environmental parameters by a thermodynamical approach. The solubility parameters will be used to optimize the preparation of gliadin nanoparticles with the aim of estimating their size.

Theory

Mixing enthalpy Δ^MH of polymer/solvent system

Flory [6] evaluated the mixing enthalpy for a regular system, i.e., for real polymer/solvent mixtures in the case of a weak thermic effect. The total energy of the interaction ΔE_{12} is a function of the elementary interaction energies of solvent/solvent (E_{11}), of polymer segment/polymer segment (E_{22}) and of solvent/polymer segment (E_{12}):

$$\Delta E_{12} = \tfrac{1}{2}(E_{11} + E_{22}) - E_{12} \,. \tag{1}$$

If z is the coordination number of polymer network and N_1 the number of moles of the solvent and ϕ_2 the volume fraction of the polymer, the mixing enthalpy is

$$\Delta^{\mathrm{M}}H = (z-2)\, N_1\, \phi_2\, \Delta E_{12} \,. \tag{2}$$

Solubility parameter δ and interaction energies

Hildebrand proposed the following relation for ΔE_{12} for endothermic "simple" mixtures:

$$\Delta E_{12} = [(E_{11}^{1/2} - E_{22}^{1/2})/2]^2 \quad (3)$$

from which the definitive form of $\Delta^M H$ is

$$\Delta^M H = V \phi_1 \phi_2 (\delta_1 - \delta_2)^2 \quad (4)$$

with V the molar mixture volume, ϕ_1 and ϕ_2 are, respectively, the volume fraction of the solvent and of the polymer.

δ_i is the solubility parameter of the compound defined as

$$\delta_i = [E_{ii}(z-2)/(2V_i)]^{1/2} \quad (5)$$

i, i.e. 1 or 2, means, respectively, the solvent or the polymer. V_i is the molar volume of the compound i. For classical solvents, the parameter can be found in the literature data [7] and is expressed in $(J\,cm^{-3})^{1/2}$ or in $(MPa)^{1/2}$.

Relation (4) shows that the miscibility of a polymer in a solvent is possible if

$$\delta_1 = \delta_2 \, . \quad (7)$$

The solubility parameter δ_M of a mixture, for instance water/ethanol, is determined with the help of the following relation:

$$\delta_M = \phi \delta + \phi' \delta' \quad (8)$$

with δ and δ' the solubility parameters of ethanol and water and ϕ and ϕ' their volume fractions, respectively.

$(\phi + \phi' = 1)$.

In this study, we are assuming that the protein/water/ethanol mixture is a simple regular mixture. In fact the protein is very diluted (0.1%).

Material and methods

Material

Gliadin was obtained from a common wheat flour (variety Hardy) and purified by INRA de Nantes laboratories, and then dialysed. Its method of purification is as follows:

Gluten was extracted on a preparative scale from the wheat flour. It was freeze-dried, ground in a refrigerated grinder and defatted by two extractions with dichloromethane for 2 h at 20 °C (gluten/solvent ratio: 1/10 w/v). After filtering, the residue was evaporated from the gluten at 20 °C under reduced pressure. Samples of dried gluten powder (50 g) were stirred gently in an ethanol/water mixture (70/30 v/v) in a gluten/solvent ratio: 1/10 w/v for 4 h at 20 °C. The suspension was centrifuged (10 000 g for 20 min). The soluble fraction was dialysed exhaustively, first against water, and then against 0.05 M acetic acid. Finally, gliadin was freeze-dried.

Analysis of the extracted gliadin fraction was carried out by reverse phase-HPLC [9] and polyacrylamide gel electrophoresis at acid pH [10]. In this way, the amount of protein in the gliadin freeze-dried extract was calculated to be around 85% w/w and the proportions of the different gliadin groups were 55% w/w for α and β-gliadins, 15% w/w for γ-gliadin, and for ω-gliadin.

All aqueous solutions were prepared from ultra pure water (Milli Q Plus-Millipore). The physiological saline phase was prepared from NaCl provided by Merck.

Pure absolute ethanol at 99.5% was provided by Carlo Erba.

Pure propylene glycol (PG) or 1,2-propanediol greater than 98% was provided by Fluka.

Pure ethylene glycol (EG) greater than 99.8% R.P. was provided by Prolabo.

The surface-active agent which disperses the nanoparticles is Synperonic® PE/F68 provided by ICI.

Ethanol was eliminated by evaporation with Rotavapor R-114, provided by Büchi, under reduced pressure (Divac 2.4L provided by Leybold).

Nanoparticle separation by ultracentrifugation was carried out with the help of Sigma 3K30 (Bioblock Scientific). The solutions' turbidity was determinated by UV-visible Lambda 5 photometer (Perkin-Elmer). Particle diameters were estimated by quasi-elastic light scattering (QELS), with the help of a photogoniodiffusometer and a RTG correlator (ΣEMATech) with 12 digital counters.

Preparation of different-solubility-parameter solutions

According to Hildebrand, the solubility parameter of gliadin can be determined using a panel of solvents or mixtures of them. The solvent which best solubilizes gliadin has the same solubility parameter (δ_G) as this protein.

Different mixtures were prepared with solvents such as ethylene glycol (EG) or propylene glycol (PG) and ultra pure water. Table 1 displays the solubility parameters of the solvents used [7].

0.1% gliadin solutions were prepared in solvents mixtures where δ_M was chosen between 30.2 and 36.9 $MPa^{1/2}$.

Preparation of gliadin nanoparticles by desolvation method

This method is based on the fact that gliadin is not soluble in a non-solvent medium such as water, in contact of which the protein precipitates.

Table 1 Solubility parameters of the solvents used

Solvents	Water	Ethylene glycol	Propylene glycol	Ethanol
δ ($MPa^{1/2}$)	47.8	32.9	30.2	26.5

Two solutions prepared separately were mixed. The method is as follows:

- 0.5% (w/v) Gliadin is solubilized at 25 °C in 20 ml of an ethanol/water mixture A of required δ_M, prepared and then filtered on 0.45 μm.
- Solution B is made from 40 ml of physiological saline solution (i.e. 0.9% NaCl aqueous solution), filtered on 0.45 μm, and from Synperonic® PE/F68 at 25 °C. The surface-active agent concentration is 0.5% w/w.
- The solution B is slowly poured with the help of a funnel, 4 mm in diameter, diving into the solvent phase A at 25 °C, under magnetic stirring (500 r.p.m) for about 10 min. The stirring and the temperature are maintained for 5 min after this operation. We call this coacervation step of the nanoparticles "*nanoprecipitation*".
- The suspension of nanoparticles is concentrated by *evaporation* with a rotavapor in order to eliminate most of the ethanol.
- Then the nanoparticles are purified by *centrifugation* for 15 min at 20 000 r.p.m and at 4 °C. The surpernatant is removed and then the pellets are resuspended in 2 ml of supernatant by three sessions of 5 min ultrasonic waves.

The different steps of the preparation are described in Fig. 1.

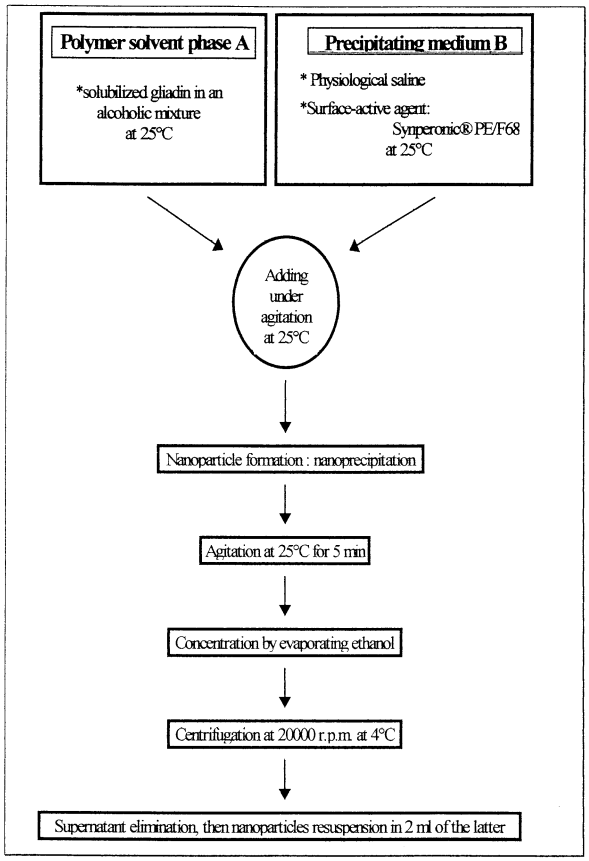

Fig. 1 Preparation of gliadin nanoparticles by desolvation method

Cross-linking of gliadin nanoparticles

This stage is reached by adding glutaraldehyde to the suspension. Freshly prepared particles were cross-linked with 1.64 mg glutaraldehyde/mg nanoparticles for 2 h at room temperature. This colloidal system was then purified twice by centrifugation in physiological saline solution.

Characterization of desolvation method media and of gliadin nanoparticles

Turbidity

For the determination of its solubility parameter, the solubility of a compound dissolved in media of different δ_M is usually inspected visually for homogeneity and transparency of the mixtures. More accurate measurement can be made by turbidity experiments. The more opaque the solution, the more attenuated the intensity of the incident beam. The observed absorption is related to the turbidity τ and to the thickness of the cell l.

The measurement is performed by determining the difference between a suspension and the pure solvent:

$$(\mathrm{Abs})_{1+2} - (\mathrm{Abs})_1 = l(\tau_{1+2} - \tau_1) = l\Delta\tau \quad (9)$$

with $(\mathrm{Abs})_i = \log(I/I_0)$; the subscripts 1 and 2 denote solvent and solute, respectively, in this case gliadin.

Quasielastic light scattering (QELS)

Particle-size measurements are carried out using quasielastic light scattering (QELS). By this method, mean particle diameters and sample polydispersity are determined. This technique is concerned with the time dependence of the fluctuations on the intensity of light scattered by a suspension of nanoparticles. The correlator accumulates the value of the light correlation function in 12 digital counters. Before an analysis, the sample is warmed up at 25 °C for, at least, a quarter of an hour; each change of one

degree centigrade promotes an error of 2% on the size estimation.

For a system of rigid monodisperse particles, the correlation function is

$$C(t) = A\exp(-2\Gamma t) + B \quad (10)$$

with A and B constants and t the measurement time. $C(t)$ is related to the fluctuation relaxation time τ by

$$\Gamma = Dq^2 = 1/\tau \quad (11)$$

where q is the scattering fluctuation wave vector amplitude. D is the translational diffusion coefficient which allows the determination of the size of a particle at high dilution limit through the Stokes–Einstein equation by extrapolation at zero volume fraction:

$$D = kT/3\pi\eta d \quad (12)$$

with k the Boltzmann constant, T the absolute temperature and η the viscosity of the medium in which the particles of hydrodynamic diameter d are suspended. Particle size measurements are performed with diluted solutions close to the dilution limit. And, for spherical particles, only scattering at one angle, 90 °, is required. In our case, the gliadin nanoparticles had been shown spherical by SEM [5].

Experimental results

Determination of gliadin solubility parameter, δ_G (Hildebrand's method)

The visual observation of 0.1% gliadin solutions gives the results shown in Table 2. The solvents used are usually considered as references for the determination of solubility parameters.

This first approach leads to the conclusion that the solutions are clearer between 30.2 and 36.9 $Mpa^{1/2}$. Then the gliadine solubility parameter, $\delta_{G'}$ is located at 35 ± 1 $MPa^{1/2}$.

To improve and quantify this result, a turbidity study has been performed at 25 °C with similar ethanol/water mixtures of δ_M from 34.0 to 36.1 $Mpa^{1/2}$. This couple of solvents was chosen because it will concern the preparation of gliadin nanoparticles. Figure 2 shows the obtained results.

It can be concluded more precisely that δ_G value is 34.5 ± 0.5 $MPa^{1/2}$.

Gliadin nanoparticle size by QELS

In a first step, the nanoparticles not reticulated were studied.

Table 2 Visual observations of 0.1% gliadin solutions

δ [$MPa^{1/2}$]	Mixture [%]	T [°C]	Appearance
30.2	100 PG	14.6	Very cloudy
30.6	96 PG/4 Water	14.6	Cloudy
31.8	91 PG/9 Water	14.6	Very cloudy
32.8	85 PG/15 Water	14.6	Very cloudy
32.9	100 EG	17.2	Cloudy
33.9	79 PG/21Water	14.6	Clear
34.2	91 EG/9 Water	17.2	Very clear
35.0	73 PG/27 Water	14.6	Very clear
35.1	85 EG/15 Water	17.2	Very clear
36.0	79 EG/21 Water	17.2	Clear
36.9	73 EG/27 Water	17.2	Cloudy

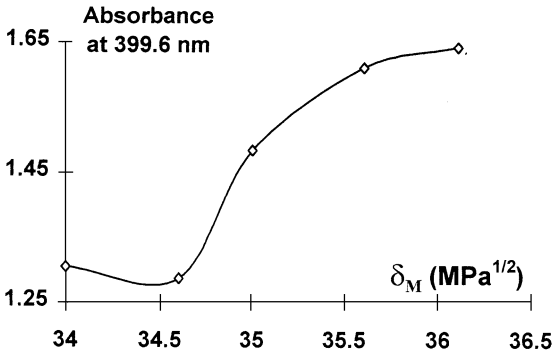

Fig. 2 Turbidity of gliadin mixtures

This study is based on three different batches of nanoparticles. Each shown diameter is an average of, at least, three measurements by QELS.

Evolution of nanoparticles with the process step

Figure 3 shows the nanoparticle diameters plotted versus the solubility parameters δ_M of the solvent mixtures used for the desolvation, 24 h after the desolvatation process.

The curves show a minimum size of the diameter when δ_M corresponds to the gliadin solubility parameter δ_G for the steps of nanoprecipitation and evaporation by rotavapor. This minimum size has been observed in three different sets of experiments. For the centrifugation step, the behavior is not the same: an inflexion point can be observed at δ_G on the decreasing plot. As shown by Table 3, the size is doubled with respect to the nanoprecipitation step at δ_G.

A similar observation is noted, in Fig. 3, between 32.9 and 35.0 $Mpa^{1/2}$. A possible explanation could be the aggregation of the particles in both by the action of the centrifugal force. But, from 36.1 to 37.9 $Mpa^{1/2}$, diameters

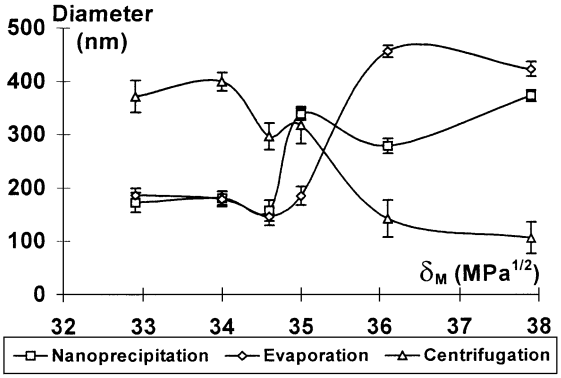

Fig. 3 Size evolution of gliadin nanoparticles as a function of the process step, 24 h after desolvation. The error bars represent the polydispersity of the size distribution

Table 3 Minimum size of gliadin particles 24 h after desolvation

Process stage	Nanoprecipitation	Rotavapor	Centrifugation
Diameter (nm)	157.4	146.2	296.4

decrease and the polydispersity values increase between 20 and 35%. This different behavior is not easy to explain. None of the parameters or experimental conditions were changed during the experiments.

Nanoparticle size as a function of δ and time

Figure 4 shows the evolution of the nanoparticle size from 24 h after their nanoprecipitation to 39 days after ($d + 39$).

During the step of nanoprecipitation, the nanoparticles were subjected neither to evaporation, nor to centrifugation. This plot and Fig. 5 show that, after 28 days ($d + 28$), the graphs are not complete. In these plots, several points are missing since $d + 28$. In fact flocculation is achieved and the colloidal concentration is too small to be measured by QELS.

Figure 5 shows the evolution of nanoparticle size from 24 h after their evaporation until 39 days after ($d + 39$). These particles were not subjected to centrifugation.

Figures 4 and 5 show a similar shape of the curves for nanoprecipitation and solvent evaporation stages with a minimum diameter obtained in the vicinity of δ_G. As for the general shape of the graphs, time does not seem to have a great influence on the minimum size. Nevertheless, for a given δ_M different from δ_G, the mean diameter of the nanoparticles changes with time.

The centrifugation (Fig. 6) has three effects: it affects the size of particles after the nanoprecipitation, and influences the temporal size of the suspension, and increases the polydispersity of size distributions. However, in the area of solubility parameters around δ_G, the evolution of the nanoparticle size is less important. Then a greater stability of size at δ_G can be deduced.

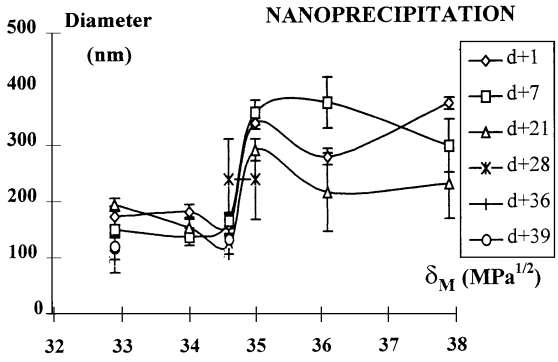

Fig. 4 Evolution of nanoparticle size after precipitation as a function of δ_M and time

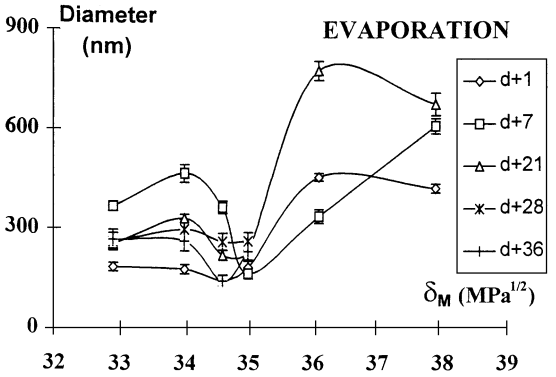

Fig. 5 Evolution of nanoparticle size after precipitation and after evaporation by rotavapor as a function of δ_M and of the time. The symbols are the same as in Fig. 4

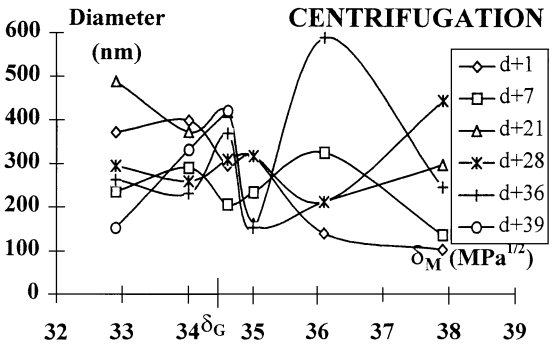

Fig. 6 Evolution of nanoparticle size after precipitation, solvent evaporation and centrifugation as a function of δ_M and time. The symbols are the same as in Fig. 4

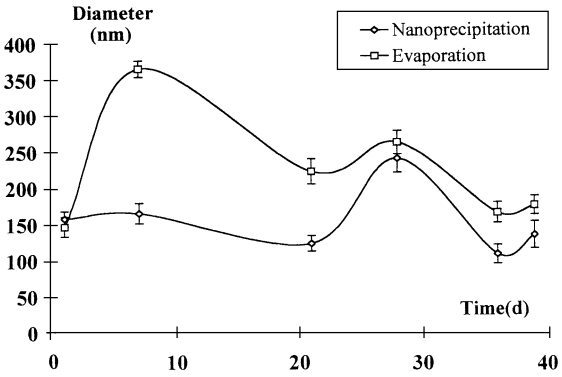

Fig. 7 Size evolution of gliadin nanoparticles ($62EtOH/38H_2O$) in time after nanoprecipitation and rotavapor

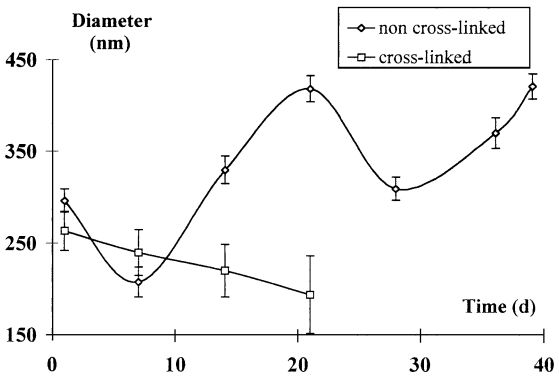

Fig. 8 Time evolution of gliadin nanoparticles cross-linked or not after centrifugation

Nanoparticle size as a function of time

The former studies show a minimum and rather "stable" size at δ_G. A comparison of the evolution of the nanoparticles diameter prepared at δ_G after nanoprecipitation and after solvent evaporation follows.

Figure 7 displays a similarity in the relationship between the size evolution and time. Nevertheless, after the rotavapor stage, which eliminates most of ethanol, the size becomes more important than after nanoprecipitation. This is probably correlated to the higher concentration of nanoparticles, which are brought closer to each other and tend to aggregate. It can be also observed that the difference between the two curves fades away in time. After 40 days, the size is approximately the same as that 24 h after the preparation of nanoparticles.

Compared evolution of the size of cross-linked and non-cross-linked particles in time

To prevent any size variations in time, a set of nanoparticles was cross-linked after the process of centrifugation and re-suspended in physiological saline.

Two interesting observations can be made from Fig. 8: the size of the non-cross-linked particles oscillates with time and their mean size increases also with time.

Contrarily to what might be expected [5], the size of the cross-linked nanoparticles reduces with time and the solution flocculates. The glutaraldehyde is known to react with the NH_2 groups of the protein. From this, it may be assumed that the charge of such groups in aqueous solution is neutralized by glutaraldehyde and, consequently, on one side, the protein is more confined, then the size decreases. On the other side, the suspension flocculates by lack of electrostatic repulsions between nanoparticles, because all NH_3^+ groups were changed into NH_2 ones.

Conclusion

This study shows that the method of the formation of gliadin nanoparticles by the solubility parameters allows the control of their size. Indeed, when the gliadin is solubilized in its good solvent, whose solubility parameter is as that of the protein, the smallest size of the particles is obtained whatever the conditions of protein extraction and purification. This size varies with the different stages of the process, the most disturbing of which being centrifugation. Time, and chemical cross-linking affect the evolution of particle size.

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