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Gliadin nanoparticles for the controlled release of all-*trans*-retinoic acid

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Abstract

The general objective of this work was to study the feasibility of preparing small-sized carriers from vegetal macromolecules. For this purpose, gliadin (a vegetal protein fraction from wheat gluten) nanoparticles were chosen as drug carriers for all-*trans*-retinoic acid (RA). The systems were prepared by a desolvation method for macromolecules, which enabled us to obtain gliadin nanoparticles of about 500 nm, with a yield close to 90% of the initial protein. All experiments were performed using environmentally acceptable solvents such as ethanol and water. Moreover, due to the low solubility of this protein in water and to its high hydrophobicity, nanoparticles from gliadin do not need any further chemical or physical treatment to harden them. Gliadin nanoparticles were quite stable over 4 days in phosphate-buffered saline (PBS), but were degraded rapidly over 3 h when incubated in PBS solution containing trypsin. However, chemical cross-linkage of nanoparticles with glutaraldehyde significantly increased their stability. Under our experimental conditions, the payload limit was 76.4 μg RA/mg nanoparticles (for an RA/initial protein ratio of 90 μg/mg), which corresponded to a RA entrapment efficiency of about 75% of added drug. Nevertheless, the entrapment efficiency was high (between 97 and 85%) for RA/initial protein ratios up to 90 μg/mg. Finally, the in vitro release profiles of RA-loaded gliadin nanoparticles showed a biphasic pattern. An initial burst effect (in which about 20% RA was released) followed by zero-order diffusion (release rate 0.065 mg RA/h) were observed.

Keywords: Gliadin; Nanoparticle; Desolvation; Controlled release; Cross-linking; All-*trans*-retinoic acid

1. Introduction

All-*trans*-retinoic acid (RA) appears to be involved in the proliferation and differentiation of epithelial tissues. On one hand, RA reduces the size of sebaceous glands as well as sebum secretion making it an attractive agent for the treat-

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ment of skin disorders, such as acne, psoriasis, hyperkeratosis, ichthyosis and epithelial tumors (Elder et al., 1992; Lewin et al., 1994). For acne treatment, it has been demonstrated that RA prevents inflammatory lesions by loosening follicular impactions (microcomedones) and clearing the follicular canal of retained keratin (Lavker et al., 1992).

On the other hand, RA has been proved effective against a range of malignancies in human clinical trials, but many patients relapsed after a short-lasting remission (Drach et al., 1993). In vitro, RA is a potent inducer of myeloid differentiation, both in the myeloblastic cell line HL-60 and in fresh blasts (Breitman et al., 1983; Chomienne et al., 1990). In vivo, numerous studies have shown that RA is active against acute promyelocytic leukemia (Huang et al., 1988; Castaigne et al., 1990; Seiter et al., 1995). Unfortunately, recent evidence has indicated that the basis for the limited duration of RA activity, at least in acute promyelocytic leukemia, is a pharmacological adaptation resulting in reduced serum concentration after prolonged treatment (Delva et al., 1991).

In addition, it has been reported that RA is transported, stored and distributed to cellular targets by specific carrier proteins, i.e. retinoic acid-binding protein (Siegenthaler and Saurat, 1987a). Furthermore, in these cells, some cytoplasmic proteins (i.e. cellular retinoic acid-binding protein) regulate the effective concentration of RA which reaches the nucleus at a level appropriate for the differential regulation of gene transcription (Siegenthaler and Saurat, 1987b).

However, in spite of the therapeutic interest of this drug, several drawbacks and undesirable effects (i.e. teratogenicity) have been reported for the currently available dosage forms (Lewin et al., 1994). To overcome these inconveniences, and in an attempt to increase the therapeutic efficacy of RA, alternative dosage forms have been suggested, including microemulsions (Takino et al., 1994) and liposomes for intravenous (Mehta et al., 1994) and topical administration (Masini et al., 1993). Another system suitable for controlled drug release could be nanoparticles from biodegradable polymers. For this purpose we have chosen nanoparticles from gliadin, a vegetal protein fraction from gluten.

Nanoparticulate carriers from vegetal macromolecules are a new approach which may present some advantages. Proteins are metabolizable and they can incorporate a wide variety of drugs in a relatively non-specific fashion (Kramer, 1974). Moreover, it has been reported that some exogenous proteins (e.g., gliadin from wheat gluten) are able to interact with epidermal keratin of the skin by means of weak but numerous bonds (Teglia and Secchi, 1994).

Wheat gluten is a protein carbohydrate complex of which proteins are the major component. Two main fractions are present: gliadin, which is soluble in neutral 70% ethanol, made of single-chain polypeptides with an average molecular weight of 25–100 kDa linked by intramolecular disulphide bonds; and glutenin, an alcohol-insoluble fraction consisting of gliadin-like subunits stabilized by intermolecular disulphide bonds in large aggregates with molecular weight greater than 106 kDa (Bietz and Rothfus, 1970). Thus, the term gliadin defines a group of proteins extracted from gluten by 70% ethanol (Kasarda, 1980). These proteins are polymorphic and can be classified on the basis of their electrophoretic mobility in four fractions, named alpha (molecular mass about 25–35 kDa), beta (30–35 kDa), gamma (35–40 kDa), and omega (55–70 kDa), respectively (Du Cros and Wrigley, 1979; Byers et al., 1983; Larré et al., 1991). All fractions have remarkably low solubility in aqueous solution except at extreme pH. This low water solubility has been attributed to the presence of interpolypeptide S–S bonds and to the co-operative hydrophobic interactions which cause the protein chains to assume a folded shape. The amino acid composition shows that gliadin has equal amounts of apolar and neutral amino acids, mainly glutamine (about 40%). Furthermore, gliadin also presents a high proline content (14%) and a very low proportion of charged amino acids (Byers et al., 1983; He et al., 1992).

The aim of this work was to prepare and to characterize gliadin nanoparticles. In addition, the in vitro capacity of these systems as carriers for all-*trans*-retinoic acid was evaluated.

2. Materials and methods

2.1. Materials

All-*trans*-retinoic acid (RA), glutaraldehyde (25% aqueous solution), and trypsin type I from bovine pancreas were obtained from Sigma Chemical Company (St. Louis, USA). Synperonic PE/F 68 was purchased from I.C.I. (Kortenberg, Belgium). Organic solvents (acetone, methanol, ethanol, propan-1-ol and propan-2-ol), sodium chloride and other chemicals used to prepare phosphate buffer were of analytical grade and obtained from Prolabo (Paris, France).

2.2. Methods

2.2.1. Gliadin extraction and purification

Gluten was extracted on a preparative scale from a common wheat flour (variety Hardi). 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). After filtration, residual solvent 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; gluten/solvent ratio: 1/10) for 4 h at 20°C. The suspension was centrifuged (10 000 × *g* for 20 min). The soluble fraction, consisting of gliadin, 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 (Wieser et al., 1994) and polyacrylamide gel electrophoresis at acid pH, A-PAGE (Larré et al., 1991) (data not shown). 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 alpha- and beta-gliadins, 15% w/w for gamma-gliadin, and 15% w/w for omega-gliadin.

2.2.2. Preparation of gliadin nanoparticles

Empty and RA-loaded gliadin nanoparticles were prepared by a desolvation method previously described (Fessi et al., 1986). Briefly, 100 mg of protein was dissolved in 20 ml of an organic solvent/water phase. This solution (organic phase) was then poured into 40 ml of a constantly magnetically stirred (250 rpm) physiological saline phase (NaCl 0.9% w/v in water) containing 0.5% w/v Synperonic PE/F 68 as stabilizer. The organic solvent was then eliminated by evaporation under reduced pressure (Büchi RE-140, Switzerland). Gliadin nanoparticles thus prepared were purified by centrifugation (Beckman J2-21M/E centrifuge equipped with a J 20.1 rotor) at 20 000 rpm for 15 min. The supernatant was removed and the pellets were resuspended in saline solution (NaCl 0.9% w/v). This suspension was centrifuged again, and finally the gliadin nanoparticles were kept in 10 ml phosphate buffered saline (PBS; pH 7.4, ionic strength 0.15 M) or dried under vacuum at room temperature and stored in a desiccator at 4°C.

To optimize the nanoparticle preparation other organic phases were tested. Thus, the following organic solvent/water mixtures were assessed: ethanol/water (7/3 v/v), methanol/water (8/2 v/v), acetone/water (5/5 v/v), propan-1-ol/water (5/5 v/v), and propan-2-ol/water (5.5/4.5 v/v).

Furthermore, some samples were cross-linked with glutaraldehyde to determine the influence of cross-linking on the physico-chemical characteristics of nanoparticles. Ten millilitres of gliadin nanoparticle suspension in PBS, freshly prepared, was cross-linked with 0.33, 0.82 and 1.64 mg glutaraldehyde/mg nanoparticles for 2 h at room temperature. These colloidal systems were then purified by centrifugation (two washing steps with PBS).

Finally, to prepare RA-loaded gliadin nanoparticles the organic phase was constituted by an ethanol/water (7/3 v/v) mixture, which contained the drug. The nanoparticle preparation procedure was performed in the dark (in order to avoid RA degradation), as described above.

2.2.3. Physico-chemical characterization of nanoparticles

The size of the gliadin nanoparticles was determined by photon correlation spectroscopy using a Coulter N4MD submicron particle analyzer (Coultronics, Margency, France). The size and shape of the nanoparticles were also examined

using scanning electron microscopy (SEM) in a JEOL 840 instrument (Germany).

The surface properties of the gliadin nanoparticles were analysed by determining their zeta potential on a Malvern Zetasizer 4 (Malvern Instruments, France) in physiological saline solution.

The amount of gliadin transformed into nanoparticles was determined as follows: 5 ml of suspensions were centrifuged (20 000 rpm for 15 min) and the residue was digested with an ethanol/water (7/3 v/v) mixture at room temperature for 10 min. The samples were measured in a spectrophotometer Spectronic 601 (Bioblock Scientific, Illkirch, France) at 280 nm. In preliminary experiments, absorbance at 280 nm had been shown to depend only on the concentration of the protein and to be linear up to 2 mg gliadin/ml.

2.2.4. In vitro degradation studies

Empty gliadin nanoparticles were suspended in PBS (pH 7.4, ionic strength 0.154 M, 0.02% sodium azide as preservative) with or without trypsin (1000 BAEE units/ml). The final concentration of the nanoparticle suspensions was 1 mg/ml. Vials containing these systems were placed in a shaking bath at 37°C. At certain time intervals, the particles were separated by centrifuging (Beckman J2-21, USA) at 20 000 rpm for 15 min. The supernatant was removed and replaced with saline solution (NaCl 0.9% w/v). After re-centrifugation, the supernatant was decanted and the particles remaining were digested in 3.0 ml of ethanol/water (7/3 v/v) solution for 10 min (non-hardened particles) or overnight (cross-linked particles). The degradation of gliadin nanoparticles was estimated by comparing UV absorbance at 280 nm of the digested nanoparticle suspensions with the absorbance obtained after digesting initial nanoparticle controls.

2.2.5. RA HPLC analysis

A liquid chromatograph (Beckman Instruments, Berkeley, USA) equipped with a 20 μl sample loop injector was used. RA was analyzed by using a modification of an isocratic procedure previously reported (Furr et al., 1992). Samples were chromatographed using a 15 cm × 4.6 mm i.d. stainless-steel column packed with octadecyl silane C18 (Ultrasphere ODS, 5 μm, Beckman) and a flow rate of 1.8 ml/min. The mobile phase, filtered through a 0.45 μm filter and degassed prior to use, consisted of methanol and water containing 10 mM ammonium acetate (75/25 v/v). The column effluent was monitored using a UV detector set at 350 nm.

RA stock solutions in methanol were refrigerated and kept in the dark. Calibration curves were constructed from the methanolic RA stock solutions over the range 0.5 to 60 μg/ml. The curves were linear and passed through the origin ($r^2 = 0.998$; $n = 6$). The repeatability (variation coefficient) was determined to be 0.45% for the 60 μg/ml RA concentration. Finally, the detection limit was calculated to be 0.2 μg/ml.

2.2.6. RA loading

RA-loaded gliadin nanoparticles (1–2 mg) were digested in 10 ml of an ethanol/water mixture (7/3 v/v) at room temperature in the dark. The samples were then assayed for drug content by UV spectroscopy at 350 nm. Although gliadin molecules did not interfere, empty nanoparticles were treated in the same way and used as references for these determinations. Moreover, supernatants obtained from the two washing steps were analysed by the HPLC method described above. This technique allowed determination of the amount of RA not associated with gliadin nanoparticles.

The drug loading (payload) and the entrapment efficiency were calculated as follows:

$$\text{Payload (\%)} = \frac{\text{amount of RA in nanoparticles (mg)}}{\text{gliadin nanoparticle yield (mg)}} \times 100 \quad (1)$$

$$\text{Entrapment efficiency (\%)} = \frac{C_1}{C_1 + C_2} \times 100 \quad (2)$$

where C_1 is the amount of RA loaded in nanoparticles (determined from spectrophotometric experiments), and C_2 is the amount of free RA (data from HPLC measurements).

2.2.7. In vitro drug release

About 15 mg of the gliadin nanoparticles (containing 0.85 mg of RA) were suspended in 100 ml of PBS pH 7.4. To prevent drug loss due to photodegradation during the dissolution study, the dissolution assembly was protected from light. The medium was maintained at 37°C and stirred at 200 rpm. Aliquots of 1 ml were collected, at successive time intervals, and centrifuged for 15 min at 20 000 rpm. These samples were immediately analyzed by HPLC, using the procedure described above, for their RA content (each measurement was made in triplicate). Finally, in order to avoid possible thermal degradation of RA during the experiments, the in vitro drug release tests were performed for only 3 h.

3. Results and discussion

3.1. Gliadin nanoparticle preparation

The method used to prepare gliadin nanoparticles in this study is based on the desolvation of macromolecules by adding a solvent phase of the protein to a non-solvent phase. It was reported that both solvent and non-solvent phases must have low viscosity and high mixing capacity in all proportions (Puisieux et al., 1994). Therefore, the first step was to choose the solvent phase which would dissolve gliadin. For this purpose different organic solvent/water mixtures, described in the literature as being able to extract gliadin from gluten (Byers et al., 1983), were tested. The non-solvent phase was always an aqueous solution containing NaCl 0.9% w/v and Synperonic PE/F 68. Preliminary experiments had showed that the presence of a neutral salt is necessary to increase the yield of gliadin nanoparticles (data not shown).

The influence of the organic solvent/water mixture (solvent phase of gliadin) used during the preparation process on the mean nanoparticle size is illustrated in Fig. 1. It was especially interesting that the size of gliadin particles depends on the type of solvent used to dissolve gliadin. Thus, the acetone/water (5/5 v/v) and ethanol/water (7/3 v/v) mixtures enabled us to obtain both smaller particle sizes and reproductive results. Taking into account these data and the toxicity data for organic solvents, the ethanol/water (7/3 v/v) mixture was chosen to prepare gliadin nanoparticles.

Finally, it is necessary to note that gliadin nanoparticles prepared in this way were stable and further treatment by heat or chemical cross-linking was not necessary to stabilize them. However, some gliadin batches were hardened with glutaraldehyde because it is known that the stability and the drug release characteristics of microparticles and nanoparticles from proteins can be modulated by cross-linkage (Davis et al., 1987; Rubino et al., 1993).

3.2. Physico-chemical characteristics of gliadin nanoparticles

The main physico-chemical characteristics of the different batches of gliadin nanoparticles are summarized in Table 1. It was apparent that no significant change in nanoparticle size, yield and zeta potential occurred during the cross-linking step with glutaraldehyde. Moreover, morphological characterization of nanoparticles by SEM shows spherical-shaped particles (Fig. 2), whose size ranged around 500 nm. These results are similar to those found by photon correlation spectroscopy (Table 1).

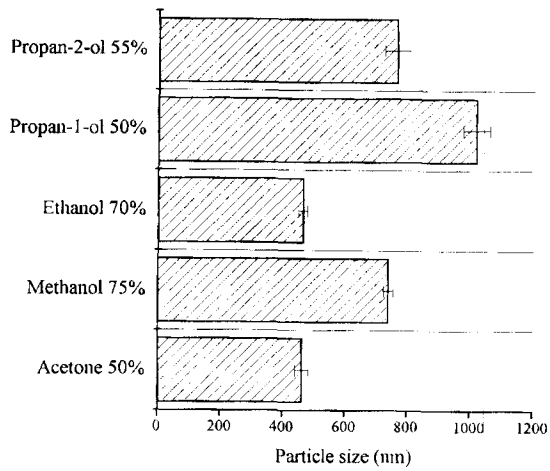

Fig. 1. Influence of the protein solvent phase (organic solvent/water mixture) on the size of gliadin nanoparticles. Experimental conditions: room temperature, $n = 4$.

Table 1
Physico-chemical characteristics of different gliadin nanoparticle batches. Data expressed as mean ± standard deviation

G/NP ratio[a] (mg/mg)	Particle size (nm)	Yield (%)	Zeta potential[b] (mV)
0[c]	464 ± 14	90.09 ± 2.20	−3.5 ± 0.15
0.33[d]	483 ± 20	89.31 ± 2.85	−3.6 ± 0.22
0.82[d]	459 ± 22	89.84 ± 3.94	−3.3 ± 0.25
1.64[d]	477 ± 19	91.03 ± 2.77	−3.4 ± 0.17

[a]Glutaraldehyde/gliadin nanoparticle ratio.
[b]Determinations in physiological saline.
[c]$n = 6$.
[d]$n = 3$.

On the other hand, the desolvation method typically yielded values close to 90% for the initial macromolecule converted into gliadin nanoparticles. Under the experimental conditions, gliadin nanoparticles were only slightly negatively charged. Their surface charges (expressed as zeta potential in mV) were found to be close to −3 mV. These results are in agreement with the low proportion of charged amino acids contained in gliadin molecules (He et al., 1992).

3.3. *In vitro stability of gliadin nanoparticles*

Fig. 3 illustrates the degradation of gliadin nanoparticles in PBS (pH 7.4; ionic strength 0.154 M; sodium azide 0.02% w/v as preservative). The nanoparticles exhibited good stability in PBS, and under these conditions, no apparent differences between non-treated and glutaraldehyde cross-linked (1.64 mg glutaraldehyde/ mg nanoparticles) particles were found. After incubation at 37°C for 4 days, only about 10 and 15%, respectively, of the non-hardened and cross-linked gliadin nanoparticles were degraded in PBS.

We also investigated the cleavage of gliadin nanoparticles in PBS solution containing trypsin (1000 BAEE unit/ml). Under these experimental conditions, the rate of degradation for all nanoparticle batches was greatly increased (Fig. 3). Indeed, for non-hardened particles, only 12% of the initial amount remained after a 3 h incubation. Similarly, gliadin nanoparticles treated with glutaraldehyde also showed a large increase in their degradation rate, since only about 40% of the initial amount remained after 6 h of incubation. However, treatment of nanoparticles with glutaraldehyde resulted in a significant decrease in the degradation rate of these colloidal systems ($p < 0.05$).

3.4. *Retinoic acid-loaded gliadin nanoparticles*

The ability of gliadin nanoparticles to carry a drug was evaluated using all-*trans*-retinoic acid (RA) as a model drug. For these experiments, only non-hardened gliadin nanoparticles were studied.

The amount of non-entrapped drug was determined by the reverse phase HPLC technique, which has been widely used for analysis of RA and other retinoids (Furr et al., 1992). This technique requires the addition of a salt, such as ammonium acetate, to the mobile phase to prevent the ionization of RA; thereby, giving better peak shapes and more reproducible retention times.

The chromatograms obtained (Fig. 4A and Fig. 4B) show the peak corresponding to RA at 8.34 min. Moreover, this technique gave an indication of the stability of retinoic acid during the preparation procedure and in vitro release studies. Indeed, we noticed the presence of a significant peak in the chromatogram at 7.8 min indicating RA degradation. In all cases, as soon as the first signs of RA degradation appeared, the studies were not continued further.

The drug content was measured by spectrophotometry after dissolving RA-loaded gliadin nanoparticles in the ethanol/water (7/3 v/v) mixture. From the results, the payload and the en-

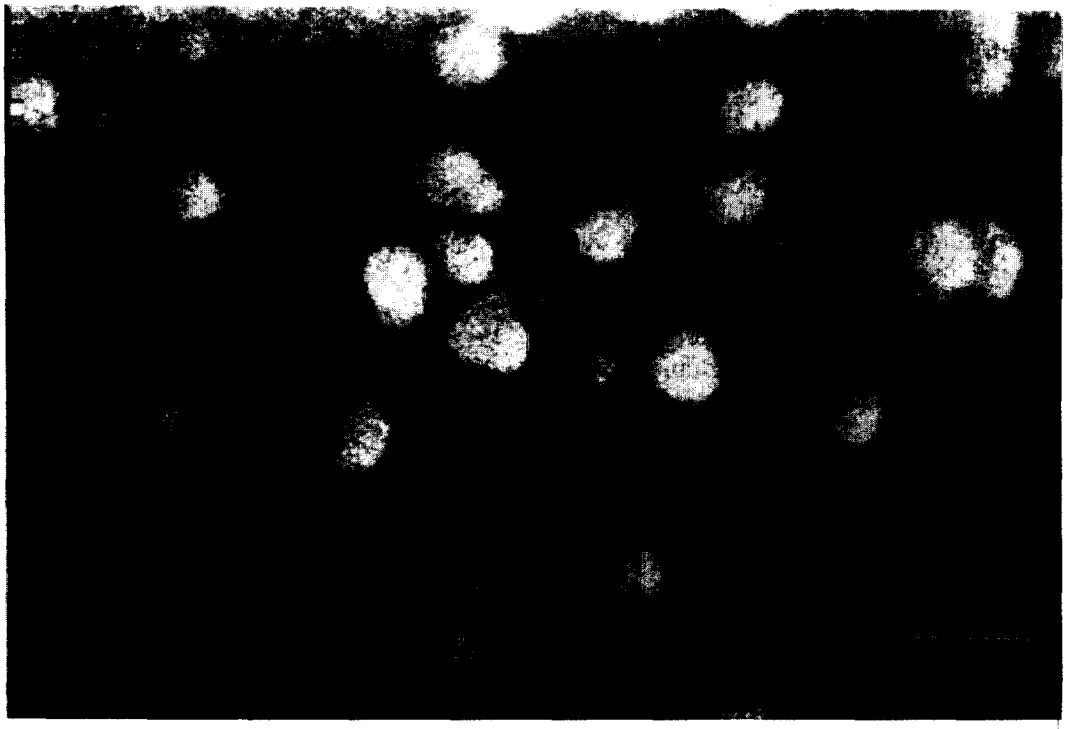

Fig. 2. SEM photomicrograph of non-hardened gliadin nanoparticles.

trapment efficiency were calculated by means of Eqs. (1) and (2), respectively (see Section 2). These two parameters were plotted against the ratio between the initial amount of RA and the initial amount of protein added to RA-loaded gliadin nanoparticles. The results are reported in Fig. 5.

Both drug loading and entrapment efficiency are affected by the drug/initial protein ratio. On one hand, the payload of RA associated with nanoparticles increased with the concentration of the drug. Although the payload could still be increased, because no plateau was obtained within the range of concentration of RA tested, the loading capacity of RA to gliadin nanoparticles was limited to ratios greater than 90 μg drug/mg protein. Under these conditions, RA precipitation and particle sedimentation occurred.

On the other hand, Fig. 5 also shows that the efficiency of the RA loading is high (between 97 and 85%) up to the level at which the drug/protein ratio was 60 μg/mg. Above this level, a large amount of unloaded RA (free RA) was present before washing steps (about 25% for a ratio of 90 μg/mg), which suggested that this free drug may be responsible for particle sedimentation. Therefore, under the experimental conditions used to prepare these gliadin nanoparticles, the limit payload was fixed at 76.4 μg RA/mg gliadin nanoparticles which corresponded to an entrapment efficiency of 75%.

3.5. Retinoic acid release from gliadin nanoparticles

Gliadin nanoparticle formulations, prepared from a drug/initial protein ratio of 60 μg/mg, were tested for in vitro release for 3 h at 37°C in the absence of light. During this period, no significant sign of RA degradation was observed. The in vitro release profiles give an indication of the efficacy of the delivery system for the controlled release of drugs. Fig. 6 shows the plot of the data expressed as the cumulative amounts of RA released from the gliadin nanoparticles as a function of time. RA was released in a biphasic way, characterized by an initial rapid and brief release

period followed by a continuous and slower release (Fig. 6). The initial release (about 15 min), usually named the burst effect, was found to be about 20% of the loaded drug. The burst effect can be attributed to the release of the drug adsorbed or entrapped in the peripheral domains of the nanoparticle matrix (Jeyanthi and Rao, 1989). On the other hand, the second slower period was approximately linear with respect to time and appeared to be a zero-order diffusion phenomenon. To confirm this type of process, the following equation was developed (Gupta et al., 1986):

$$d(Q_o - Q)/dt = -K_o \quad (3)$$

where Q is the amount of drug released, Q_o is the initial amount of drug present in the gliadin nanoparticles, t is the time during which release of RA occurs, and K_o is the rate of release which is a function of the drug diffusivity in the matrix, the carrier surface area, the drug content, and the diffusion layer thickness.

For a zero-order process, the difference between the initial amount of drug loading and the amount of drug released $(Q_o - Q)$ was plotted against the time (Fig. 6). It can be observed that this model provides an adequate fit to the data from the second period of the experiment. Thus, the rate of release (K_o) was found to be 0.065

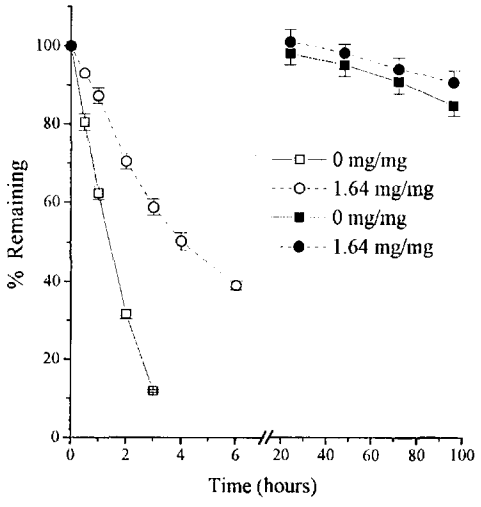

Fig. 3. Degradation of non-hardened (0 mg glutaraldehyde/mg nanoparticles; ■,□) and cross-linked gliadin nanoparticles (1.64 mg/mg; ●,○) in PBS (pH 7.4; ionic strength 0.154 M; solid symbols) and PBS containing 1000 BAEE units/ml trypsin (open symbols).

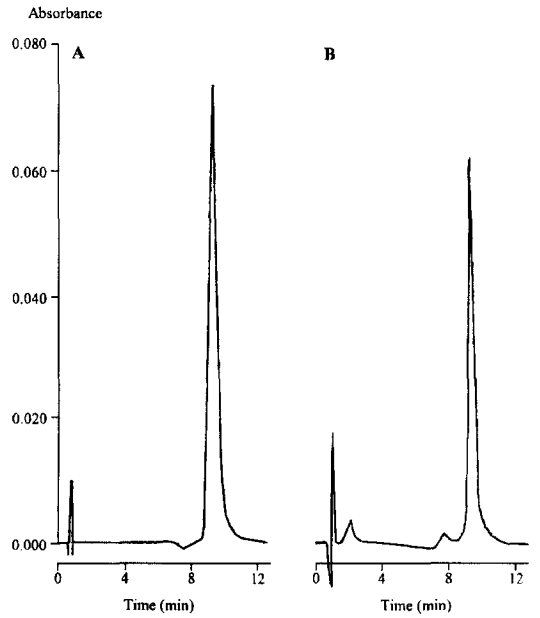

Fig. 4. Chromatograms of the standard RA in methanol (A, 30 μg/ml) and from RA in vitro release studies (B, retinoic acid released from gliadin nanoparticles after 3 h). Retention time of retinoic acid: 8.34 min.

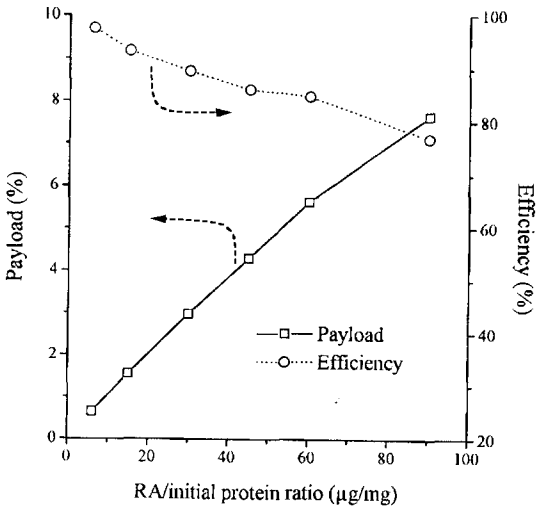

Fig. 5. Influence of the RA/initial protein ratio on the payload and the entrapment efficiency.

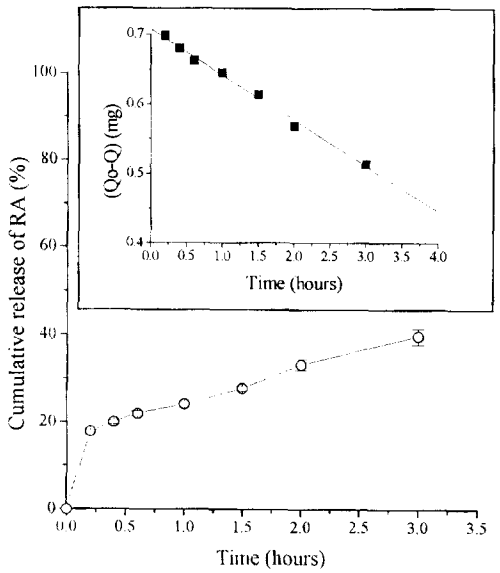

Fig. 6. In vitro release of RA from gliadin nanoparticles (expressed as cumulative release). The inset is a plot of the residual values ($Q_0 - Q$, in mg) versus time for the second period of RA release from gliadin nanoparticles.

mg/h ($r = 0.997$) for a period of 3 h. Finally, about 20% of the drug was released by diffusion during this period.

4. Conclusions

Control over the size of gliadin nanoparticles and microparticles produced by a desolvation method of macromolecules was achieved by modifying the organic solvent/water mixture used to dissolve the protein. Therefore, using only environmentally acceptable solvents such as ethanol and water (7/3 v/v), this technique allowed us to obtain reproducible particle sizes of about 500 nm with a narrow size distribution. These systems are quite stable in PBS but they are rapidly degraded in the presence of trypsin. Nevertheless, their stability in these conditions can be increased by means of chemical cross-linkage with glutaraldehyde.

When gliadin nanoparticles were assayed as carriers for RA, the limit payload was fixed to 76.4 μg drug/mg nanoparticle which corresponded to an entrapment efficiency of about 75% of added RA. Finally, the in vitro release of RA from gliadin nanoparticles was characterized by an initial burst effect (20% released) and a zero-order diffusion process for at least 3 h, in which the drug was released at a rate of 0.065 mg/h.

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