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Nisin-loaded poly-L-lactide nano-particles produced by CO_2 anti-solvent precipitation for sustained antimicrobial activity

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Abstract

Nisin-loaded poly-L-lactide (PLA) nano-particles were fabricated by processing protein/polymer organic solutions by semi-continuous compressed CO_2 anti-solvent precipitation. Preliminary solubility studies were carried out for an optimised selection of organic solvent mixtures leading to preparation of protein/polymer solutions. The particles were prepared by processing 50:50 dimethylsulfoxide/dichloromethane mixtures containing 1% polymer (w/v) and 5 or 20% nisin (nisin/polymer, w/w). Proper operative conditions (organic solution injection rate, precipitation temperature and gas pressure, CO_2 flow rate, washing time, etc.) were set up to yield production reproducibility, high product recovery (over 70%) and high drug loading (over 95% of the recovered protein). Scanning electron microscopy demonstrated that spherical, smooth surfaced particles were produced. Light scattering showed that the particle size was in the range of 200–400 nm and the products were characterised by narrow polydispersity. In vitro release studies showed that the protein is slowly released throughout 1000 h. However, the release was slower as the salt concentration and the pH of the release buffer increased. Solubility investigations suggested that the observed differences in protein release rate out of nano-particles was attributable to the protein interaction with the polymer which was found to increase as the pH or the salt concentration increased in the release buffer. In vitro studies carried out by nano-particle incubation in medium containing *Lactobacillus delbrueckeii* showed that nisin was released in the active form and the antibacterial activity was maintained up to 45 days incubation.

Keywords: Polymeric nano-particles; Antimicrobial activity; Compressed CO_2 precipitation; Protein release

1. Introduction

Lantinobiotics are a family of antimicrobial proteins of bacterial origin containing unusual amino acids such as lanthionine (Klaenhammer, 1993). These bac-

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teriocins display interesting antimicrobial activity due to their broad inhibitory spectrum against a variety of Gram-positive bacteria. Recently, FDA recognised nisin, a bacteriocin produced by *Lactococccus lactis* subsp. *lactis*, as food additive (Breukink and de Kruijff, 1999). However, the use of nisin in food preservation is strongly limited by its structural instability, deprivation by interaction with food and cell matrixes, and development of tolerant and resistant *Listeria* (Chi-Zhang et al., 2004). Therefore, excessive nisin amounts are required to guarantee effective pathogen growth inhibition.

In order to overcome these problems, several formulation approaches have been investigated. Nisin embedded packaging materials or nisin adsorbed solid surfaces such as polyvinilic or polysaccharide films allowed for prolongation of the biological activity (Sirgausa et al., 1999; Netrajan and Sheldon, 2000a, b; Coma et al., 2001; Hoffman et al., 2001; Cha et al., 2003). However, although these systems reduced nisin resistant strain development and ensured higher level of microbial safety of food products, their casting and preparation was difficult and protein inactivating. Nisin modification by attachment of poly(ethylene glycol) did not yield biologically active derivatives, though site directed conjugation was carried out, probably because the polymer attachment altered the protein structural properties (Guiotto et al., 2003). Liposome formulations were found to enhance the protein stability and allow for prolongation in biological activity (Larid et al., 2003). Nevertheless, the physico-chemical and delivery properties of these formulations were strongly affected by the electrostatic and hydrophobic nisin interactions with the liposome components. Furthermore, the liposome stability as well as the nisin release depended on the environmental conditions, being sensitive to the presence of bivalent ions or other medium components.

Nisin-loaded polymeric micro-/nano-particles seem to be promising formulations to achieve long lasting antimicrobial activity. These systems have been actively investigating as protein drug delivery systems since they can enhance the biological performance of bioactive molecules (Allémann et al., 1998; Soppimath et al., 2001; Panyam and Labhasetwar, 2003). With respect to other slow release systems, polymeric micro-/nano-colloids are physically stable and can be easily formulated with a variety of materials obtaining controlled drug release rate. Nevertheless, the fabrication of protein loaded micro-/nano-particles is limited by several problems related to the protein structural fragility and low protein loading. Aimed at reducing classical techniques drawbacks, few innovative technologies based on supercritical or gas compressed techniques have been developed to produce protein loaded polymeric nano-particles (Ruchatz and Müller, 1996; Thies and Müller, 1998; Mishima et al., 2000; Caliceti et al., 2001a, b). In particular, the limited use of technological additives such as surfactants or stabilizers which can induce protein denaturation or toxic effects, the process flexibility, the high product yield, the possibility of efficient protein encapsulation and the possibility to obtain high physico-chemical features, make these techniques particularly attractive.

The present study deals with the preparation of stable long lasting antimicrobial nisin-loaded polymeric micro-/nano-particles, which can be dispersed in food, pharmaceutical products or other materials with different physical consistence. A reproducible and scalable gas-compressed CO_2 process yielding nano-particles with homogenous properties, low particle size, low dispersity, high protein loading and efficient protein release was investigated. Poly-L-lactide (PLA) was used because it is a biodegradable and non-toxic polymer largely used in protein formulation (Anderson and Shive, 1997). Furthermore, PLA was demonstrated to possess suitable properties for compressed CO_2 antisolvent precipitation. Previous studies demonstrated that, due to its high crystallinity degree, PLA precipitates under CO_2 compressed or supercritical conditions leading to micro-/nano-particles with smooth spherical morphologies without formation of amorphous sticky precipitates (Thies and Müller, 1996; Elvassore et al., 2001). The morphological, physico-chemical and biological properties of the final products were investigated in order to verify the process reliability and reproducibility as well as the possibility to use the nanoparticles as preservatives.

2. Materials and methods

Nisin A was furnished by Aplin & Barret Ltd. (Beanmister, Dorset–England). Poly-L-lactide (L-PLA) MW 102 kDa, Resomer L 206, was obtained from Boehringer (Inghelheim, Germany). All the other

chemicals were obtained from Aldrich Chemie (Steinheim, Germany). CO_2 (99.95%) was purchased from Air Liquide (Padova, Italy).

Lactobacillus delbrueckeii spp. *bulgaricus*, MRS medium and agar culture mediums were from Difco (Sparks, MD, USA). The culture mediums were sterilized at 110 °C for 15 min in autoclave (Alpha Junior, International PBI, Milan, Italy). The sterilization process was previously set up by evaluating the micro-organism growth under aerobic and anaerobic atmosphere.

2.1. Nisin solubility in organic solvent mixtures

Nisin A organic solutions were prepared by dissolving 5, 15, 25, 35, 40 and 50 mg of protein in 10, 30, 50, 70, 80 and 90 μl of dimethylsulfoxide, respectively. The solutions were added to 90, 70, 50, 30, 20 and 10 μl of dichloromethane to reach 100 μl final volume. The mixtures were stirred and maintained for 2 h at room temperature. After centrifugation at 13,500 rpm for 5 min, 50 μl volumes were taken and dichloromethane was evaporated under mild vacuum. The residue was added of 1 ml of water containing 0.05% trifluoroacetic acid (TFA), centrifuged at 13,500 rpm for 5 min and supernatant was analysed by reverse phase HPLC using a C-18 column eluted with water/0.05% TFA (eluent A) and acetonitrile/0.05% TFA (eluent B) gradient: 0–5 min, 20% eluent B; 5–20 min from 20% eluent B to 80% eluent B. The UV detector was set at 220 nm. The nisin concentration in the aqueous solution was calculated on the basis of the peak area using a standard curve obtained with protein solutions in the range of 0.01–1 mg/ml ($y = 9342.7x - 7502.2$; R^2 0.9925).

2.2. Nano-particles production

Organic nisin/PLA solutions were processed by compressed CO_2 using the equipment described elsewhere (Elvassore et al., 2001). Protein/polyester organic solutions were prepared by dropping 20 ml of dichloromethane containing 400 mg of PLA into 20 ml of dimethylsulfoxide containing 20 or 80 mg of nisin to reach a 5/100 and 20/100 protein/polymer weight ratio (5 and 20% w/w, respectively). The organic solutions were injected at a constant flow rate of 1 ml/min and atomised through a 50 μm internal diameter fused silica capillary nozzle into a 200 ml high-pressure CO_2 precipitation vessel. The operative temperature and pressure were 288 K and 15 MPa, respectively. An electrical resistance and a heat exchanger connected to an auxiliary bath were used to achieve a constant temperature (±0.5 K) in the precipitation vessel. After precipitation due to CO_2 mass transfer into the organic solvent, CO_2 was continuously flowed through the high-pressure vessel at 1800 Nl/h rate (normal litres/h at 273 K and 1 atm) for 1 h to get rid of the residual organic solvents. Finally, the system was depressurised and the dry material was collected from the vessel bottom where a micro-filter set was previously installed. The methylene chloride and dimethylsulfoxide content in the final products was routinely estimated according to the analytical procedure reported in our previous paper (Caliceti et al., 2001a).

2.3. Particles morphology characterization

The particulate products were analysed by scanning electron microscopy (SEM) with a Cambridge Stereoscan Instrument (Cambridge, UK). All samples were maintained overnight at −20 °C and then under vacuum at 0.02 mbar for 48 h. The samples were gold sputtered (MFD 010 Bolzen Sputter) under high vacuum (0.04 mbar) and photographs were taken at magnifications ranging from 1000 to 20,000.

Dynamic light scattering analysis was carried out using a SpectraPhysics Nicomp 380 instrument (Particle Sizing Systems, Santa Barbara, CA, USA). Samples were prepared by dispersing 1 mg of product in 20 ml of distilled water. The suspensions were stirred for 5 min, sonicated for 2 min and finally analysed.

2.4. Nisin nano-particles loading evaluation

Nisin content in the final products was determined after particle dissolution and protein extraction. Five milligrams of nano-particles were dissolved under vigorous stirring in 400 μl of dichloromethane and the organic dispersion was added of 1 ml of H_2O/0.05% TFA. The two-phase mixture was stirred for 30 min and the organic solvent was left to evaporate. The samples were centrifuged at 13,500 rpm for 15 min and the solution volumes were determined. The nisin A amount in the aqueous phase was estimated by reverse phase C-18 column operated on an HPLC system as reported above.

The total protein recovery, reliability and accuracy of the method were verified by nisin A extraction from nisin/PLA physical mixtures containing known protein/polymer amounts. The nisin A recovery was found to be over 94 ± 3% with respect to the theoretical values.

2.5. Dependence of nisin solubility on pH and salt concentration

Nisin A samples of 20 mg were added of 100 μl of 0.02 M phosphate buffer, pH 5.0, containing 0.05, 0.1, 0.15, 0.2, 0.25 and 0.5 M NaCl or 100 μl of 0.02 M phosphate buffer, 0.15 M NaCl, pH 2.0, 3.0, 5.0, 6.0, or 7.4. The solutions were maintained at room temperature overnight under stirring and then centrifuged at 13,500 rpm for 30 min. The pH was checked and the nisin A content in the solutions was determined by reverse phase HPLC according to the method reported above.

Nisin A samples of 20 mg were added of 200 μl of the different buffers reported above and incubated at room temperature under stirring. At scheduled times (5, 10, 15, 20, 30, 40 and 60 min) the samples were centrifuged and the protein content in the solution was determined by reverse phase HPLC.

2.6. Evaluation of nisin/PLA interaction

Forty milligrams of unloaded PLA nano-particles were added to 1 ml of 1 mg/ml nisin A solutions in 0.02 M phosphate buffer, pH 5.0, containing 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.5 M NaCl or 0.02 M phosphate buffer, 0.15 M NaCl, pH, 2.0, 3.0, 5.0, 6.0 or 7.4. The solutions were maintained at room temperature overnight under stirring and then centrifuged at 13,500 rpm for 30 min. The nisin content in the solutions was assessed by reverse phase HPLC according to the analytical procedure reported above.

2.7. In vitro nisin release studies

Ten milligrams of nisin A loaded nano-particles were suspended in 1 ml of 0.02 M phosphate buffer, pH 5.0 containing 0.05, 0.1, 0.2, and 0.5 M NaCl or in 1 ml of 0.02 M phosphate buffer, 0.15 M NaCl, pH 2.0, 3.0, 5.0, 6.0 or 7.4. The suspensions were stirred and then sonicated for 2 min and maintained at room temperature under mild shaking. At scheduled times, the pH was checked and the samples were centrifuged at 5000 rpm for 5 min, 700 μl solutions were withdrawn and the volumes were replaced with fresh buffer. The particles were re-suspended under vigorous stirring. The 700 μl samples were lyophilised and the dry product was dissolved in 100 μl of H_2O/0.05% TFA. The nisin A content in the releasing buffer was determined by reverse phase C-18 column operated on an HPLC system as reported above.

2.8. In vitro Nisin biological activity

One milligram of free nisin or nisin equivalent nanoparticles (20 mg of 5% nisin A loaded nano-particles or 5 mg of 20% nisin A loaded nano-particles) was dissolved or suspended in 5 ml of sterile MRS medium. The medium was added of 1% *L. delbrueckeii* spp. *bulgaricus* culture medium and incubated overnight. Controls were set up by incubating 0.02 M phosphate buffer, 0.15 M NaCl (PBS), pH 7.2 and 5 or 20 mg of void PLA nano-particles. At scheduled times, 25 μl of culture medium were collected, and serially diluted in PBS (pH 7.2) to reach 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions. The withdrawn volumes were replaced with fresh medium containing 4% *L. delbrueckeii*. The diluted solutions were added to a plate containing 2% agar and incubated at 37 °C. After 24 h the colony forming units (CFU) were determined. The CFU were normalised by the corresponding dilution according to the method reported elsewhere (Caliceti et al., 2001b).

3. Results

3.1. Nano-particle fabrication set up

Solubility studies were undertaken to select proper organic nisin/PLA solutions for gas compressed CO_2 processing. The study was carried out using dimethylsulfoxide/dichloromethane mixtures, two solvents which were previously demonstrated to be suitable for preparation of insulin loaded polyester micro-particles by CO_2 anti-solvent precipitation techniques (Elvassore et al., 2001; Caliceti et al., 2004). The nisin solubility profile in dimethylsulfoxide/dichloromethane mixtures is reported in Fig. 1.

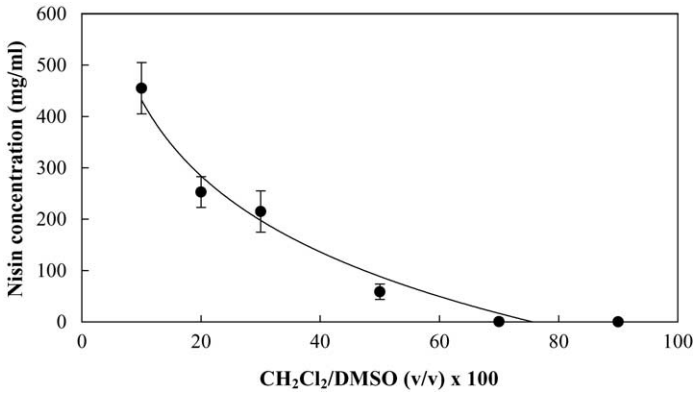

Fig. 1. Nisin solubility profile in dimethylsulfoxide/dichloromethane mixtures.

Nisin is freely soluble in dimethylsulfoxide (10 times more than in aqueous acidic solution) but its solubility decreases dramatically as the dichloromethane content in the solvent mixture increases. In particular, the nisin concentration drops less than 1 mg/ml when 30:70 dimethylsufoxide/dichloromethane volume ratio of the mixture is used.

In 50:50 dimethylsulfoxide/dichloromethane, nisin solubility was found to be about 60 mg/ml. Therefore, this dimethylsulfoxide/dichloromethane mixture was employed for preparation of stable 10 mg/ml PLA solutions containing 0.5 or 2 mg/ml of nisin (5 and 20% protein/polymer w/w, respectively).

To set up a proper operative process allowing for fabrication of nano-particles with defined and reproducible properties, nisin/PLA organic solutions were processed under different conditions: organic solution injection flow rate, precipitation temperature and pressure and CO_2 flow rate. Physico-chemical investigations were performed batch by batch to evaluate the process reproducibility and efficiency, the residual solvent content in the final product and the particle morphology properties. Optimal preparation conditions were: 1 ml/min organic solution injection into the precipitation vessel, 288 K and 15 MPa; 1500 Nl/h CO_2 flow rate during the precipitation process; and 1 h CO_2 post-precipitation washing.

3.2. Physico-chemical characterization

At least five preparative runs were carried out with each formulation under the selected operative conditions and the physico-chemical and biopharmaceutical properties of the final products were investigated.

The data reported in Table 1 indicate that similar product recovery yield, solvent content and particle size are obtained by processing the formulations containing 5 and 20% of nisin.

The process was found to yield very high product and protein recovery (about 80% of the processed material).

The dichloromethane and dimethylsulfoxide content in the final products were below 15 and 300 ppm, respectively.

The morphological features of preparations were investigated by scanning electron microscopy and light scattering. The SEM analysis showed that the particles obtained under the operative conditions presented smooth and compact surface and spherical shape. No fibres, crystals or amorphous materials were present in

Table 1
Main properties of the nisin-loaded particles: product recovery, nisin recovery and particle size

	Product recovery (recovered material/processed material, w/w %)	Nisin recovery (recovered nisin/processed nisin, w/w %)	Particle size (nm ± S.D.)
Nisin/PLA 5%	78 ± 8	81 ± 3	254 ± 29
Nisin/PLA 20%	81 ± 12	84 ± 2	387 ± 44

the final products. The light scattering data reported in Table 1 show that the products present small mean size and standard deviation (±S.D.), which indicates narrow size dispersity.

3.3. Protein release studies

Nisin release studies were performed by nano-particle incubation in buffers at various pH or containing different salt concentrations. Fig. 2 shows the nisin release profiles from 5% nisin-loaded nano-particles. The release profiles obtained with 20% nisin-loaded nano-particles did not display remarkable differences as compared to the ones obtained with the 5% nisin-loaded products reported in Fig. 2.

The amount of nisin released from the two formulations after 30 min incubation in the different buffers was in the range of 1–3% of the total nisin present in the product. This promptly available nisin was considered unloaded protein. Therefore, the protein concentration in the nano-particles was calculated to be 4.95–4.85 and 19.8–19.4% (w/w) in the case of 5 and 20% formulations, respectively.

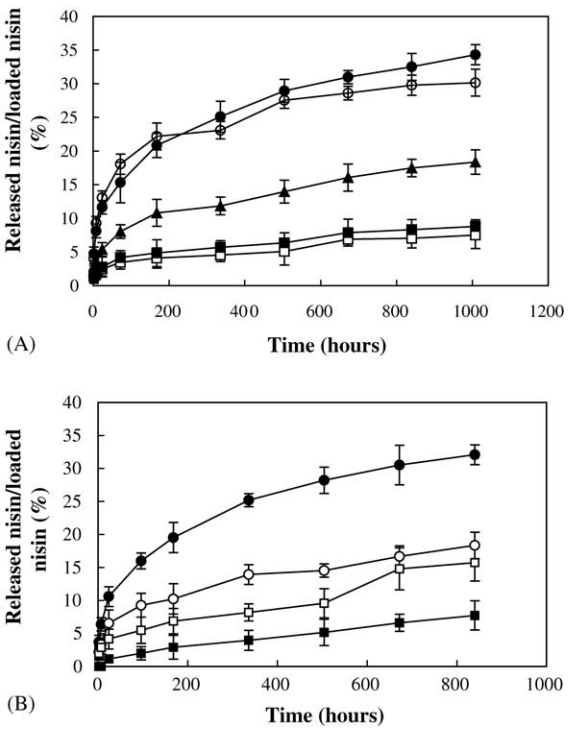

Fig. 2. Nisin release profiles in different buffers. (A) 0.02 M phosphate, 0.15 M NaCl, pH 2.0 (●), pH 3.0 (○), pH 5.0 (▲), pH 6.0 (■), pH 7.4 (□). B 0.02 M phosphate, pH 5.0, 0.05 M NaCl (●) 0.1 M NaCl (○), 0.2 M NaCl (□) and 0.5 M NaCl (■).

Fig. 2 shows that the nisin release rate decreases as the pH or the salt concentration in the release buffer increases. Furthermore, the amount of nisin released in 1000 h increased from 8% to about 30% of the loaded nisin as the pH of the release buffer decreased from 7.4 to 3.0 or the NaCl concentration decreased from 0.5 to 0.05 M. After 1000 h incubation, nisin release was negligible indicating that most of the protein was strongly entrapped into the polymeric matrices.

3.4. Nisin solubility properties

The nisin solubility, dissolution rate and interaction with the polymer in the different release buffers were investigated.

The profiles reported in Fig. 3A and B show that nisin solubility strictly depends on the buffer pH and salt concentration. In particular, the protein solubility decreases as the pH and the salt concentration increase. However, nisin is highly soluble (more than 10 mg/ml) in all the examined buffers. Also, the solubilities obtained at pH 7.4 or in 0.5 M NaCl were significantly higher as compared to the maximal concentration which could be obtained in the release studies indicating that sink conditions were maintained throughout the experiment.

Nisin dissolution rates, as fractional dissolution, determined in the different buffers were similar. Maximal protein concentration was reached in 15 min incubation.

The addition of plain PLA nano-particles (nisin unloaded) to nisin solutions decreased significantly the nisin concentration. The nisin concentration reduction was found to depend on pH or NaCl concentration in the buffer. Fig. 4 shows that the nisin concentration decreases to about 20–30% at pH > 5.0 or NaCl > 0.15 M.

3.5. Biological activity

Biological behaviour of the nisin-loaded formulations was evaluated by determining the bacterial colony forming units (CFU) after nano-particle incubation with *L. delbrueckeii*. The experiment was carried out by comparing the biological effect of nisin equivalent

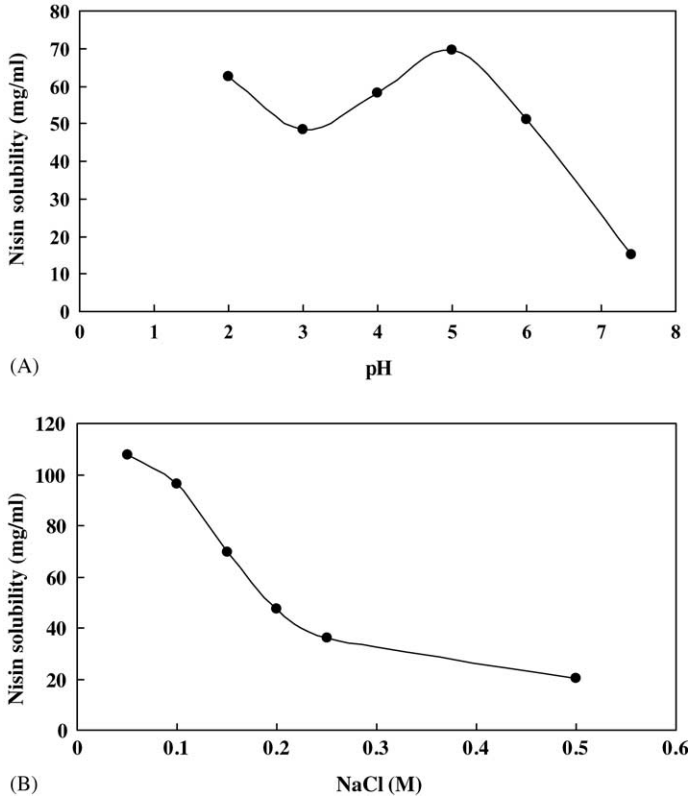

Fig. 3. Nisin solubility dependence on pH changing in 0.02 M phosphate buffers, 0.15 M NaCl (A) and nisin solubility dependence on ionic strength in 0.02 M phosphate buffers, pH 5.0 (B). The standard deviation calculated on the basis of three experiments was found to be in the range of 0.5–2% of the mean values.

amounts of nano-particles (5 or 20% nisin-loaded nano-particles) and same amount of free nisin in solution. Buffer and plain nano-particles were used as control and negative control, respectively.

The biological activity time courses obtained with the various formulations depicted in Fig. 5 are in agreement with the in vitro release studies. The two formulations, 5 and 20% nisin-loaded nano-particles, display similar behaviour and prolonged activity (up to 40 days). Free nisin samples displayed antibacterial activity for 7 days while the plain nano-particles did not have per se any antibacterial activity.

4. Discussion

The preparation of nisin-loaded polymeric nano-particles for long lasting antimicrobial activity was carried out by gas compressed CO_2 anti-solvent precipitation process. Although this technique presents some advantages over traditional ones, the preparation of polymeric micro-/nano-particles by gas compressed CO_2 anti-solvent precipitation requires specific process set up which must be pointed out according to the physico-chemical properties of the processed materials: polymer and protein.

Micro-/nano-particle formation takes place by compressed CO_2 partition into an atomised solution, which provokes solvent expansion, density decrease and solute precipitation. In order to allow for rapid and complete CO_2 partition, organic solvents with low polarity and boiling point are required. On the other hand, the selected solvents must guarantee the complete and stable dissolution of the particle components, protein and polymer to avoid trouble, some due to micro-precipitations or suspension formation into the

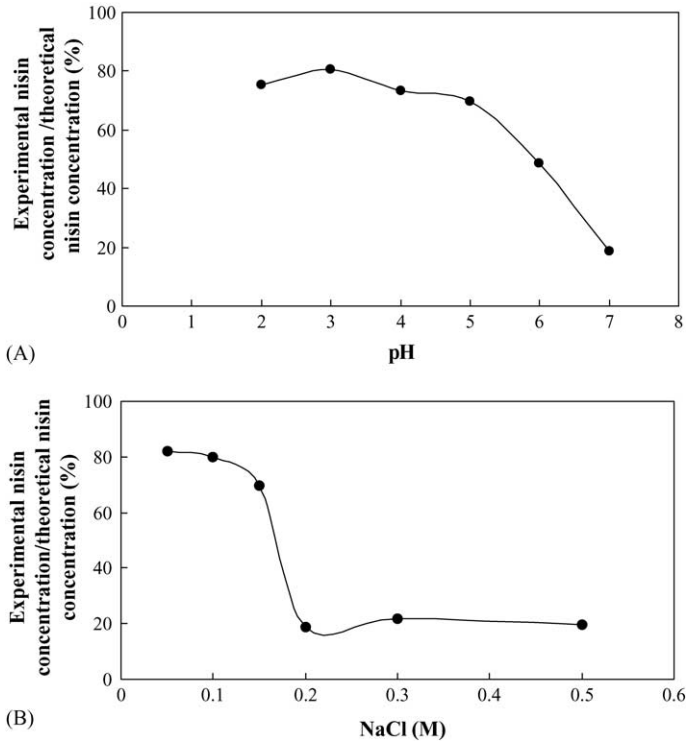

Fig. 4. Nisin solubility profiles in different buffers containing PLA plain nano-particles in 0.02 M phosphate buffers, 0.15 M NaCl (A) and nisin/NaCl concentration solubility profiles in 0.02 M phosphate buffers, pH 5.0 (B). The standard deviation calculated on the basis of four experiments was found to be in the range of 0.5–3% of the mean values.

equipment tubes. Therefore, the solvent choice represents a key point for obtaining small sized particles, namely nano-particles, where the bioactive compound is molecularly dispersed into the polymeric matrix.

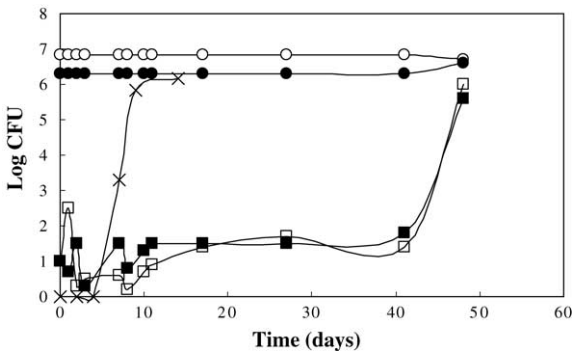

Fig. 5. Nisin biological activity time course after incubation of *Lactobacillus* with buffer (positive control, ●), plain PLA nano-particles (negative control, ○), 5% nisin-loaded nano-particles (■), 20% nisin-loaded nano-particles (□), nisin solution (×).

In the case of nisin-loaded poly-L-lactide particle preparation, the solvent choice is limited by the different solubility properties of protein and polymer being hydrophilic the former and hydrophobic the latter. Dimethylsulfoxide/dichloromethane mixtures, already successfully employed for preparation of insulin loaded PLA micro-particles (Elvassore et al., 2001; Caliceti et al., 2004), were found to be suitable to prepare nisin/PLA solutions at proper concentrations for gas compressed CO_2 processing. The dimethylsulfoxide content in the mixture should be as low as possible since the slow CO_2 partition in this solvent due to its high density and polarity leads to amorphous and sticky material precipitation with partial separation of the components, namely the polymer and the protein. Therefore, non-homogeneous particles with low protein loading and poor technological properties are obtained. On the contrary, CO_2 diffuses rapidly into dichloromethane leading to rapid solvent expansion and solute precipitation yielding shape ho-

mogenous nano-particles with high protein loading. However, the dramatic nisin solubility decrease as the dichloromethane content overcomes 50% of total volume, prevented the use of solvent mixtures containing less than 50% of dimethylsulfoxide.

The constant 1 ml/min organic solution injection rate guarantees a spray regime into the precipitation vessel, which is a requisite in obtaining nano-particles with homogeneous morphological properties. Temperature, pressure and CO_2 flow rate were selected on the basis of the processed materials. High temperature and pressure allow for rapid and extensive CO_2 mass transfer and material precipitation, but yield sticky materials due to partial polymer melting. On the other side, low temperature and pressure prevent particle fusion but decrease significantly the CO_2 partition into the organic solvents and their expansion and elimination. Therefore, temperature, pressure and CO_2 flow rate were properly balanced in order to reduce their negative effect onto the final product.

Under proper GAS precipitation conditions, final products displaying homogeneous particle size, spherical shape, extensive solvent elimination and high protein loading were obtained. The extensive solvent elimination is a requisite of primary importance for products for human application, namely food and pharmaceuticals. To note that the solvent content in the products was within the FDA and EMEA limits, which is 600 ppm for dichloromethane. The adopted process yielded very small particle size and narrow dispersity, which are usually difficult to be obtained using this technique. The small size and very low dispersity could be due to nisin/polymer interaction phenomena. Actually, studies on nisin-loaded liposome preparation demonstrated that the nisin interaction with phospholipids could affect significantly the liposome size (Larid et al., 2003). However, this unexpected result deserves further investigations, which will be carried out with proteins that can differently interact with the polymer.

Nisin release studies were carried out by nanoparticle incubation in different medium because these long lasting antimicrobial systems were supposed to be used in preservation of food or pharmaceuticals having specific and different environment. Slow but incomplete nisin release was observed throughout 1000 h. However, the studies evidenced that both amount of released nisin and release rate depended on the release conditions such as buffer pH and salt concentration. Protein solubility and dissolution studies demonstrated that, despite nisin solubility decreases significantly as the pH and NaCl content increase, sink conditions were guaranteed throughout the experimental tests. Nevertheless, the nisin concentration in solutions containing plain PLA nano-particles was found to decrease dramatically as the pH and NaCl concentration in the buffer increased indicating that nisin/polymer interactions take place. These results seem to suggest that nisin undergoes pH and ionic strength induced micro-structural arrangements, which may reflect in increased nisin hydrophobicity and, consequently, increased interaction with the hydrophobic polymer and decreased protein solubility. In particular, the dependence of nisin solubility on the pH and salt concentration and the non-linear solubility/pH correlation may be attributable to the ionisable protein immino, amino and carboxyl groups. Nisin A structure contains, in fact, two histidines, three lysines, one terminal amino group of isoleucine and one terminal carboxyl group of lysine which possess pKa 6.0, 10.53, 9.68 and 2.18, respectively. As the pH increases above 2.5 the ionisation of the carboxyl group reflects in slight protein solubility increase which is maximal at pH 5.0. Above pH 5.0, the de-protonation of the immino and amino groups induces the decreases of the protein solubility. On the other hand, ionisable protein groups are progressively masked as the salt concentration increases, which reflect in protein solubility decrease. Also, the presence of carboxyl groups generated by the polymer degradation could promote the nisin/polymer interaction. This hypothesis is supported by the fact that nisin was demonstrated to interact with lypophilic macromolecules and biological matrixes either by electrostatic or hydrophobic interactions (El Jatzumi and Lafleur, 1999). Indeed, nisin biological activity takes place by interaction with the bacteria membrane with subsequent membrane depolarisation (Demel et al., 1996). Furthermore, several studies demonstrated that this bacteriocin can assemble with lipids and phospholipids of different origin and the preparation of nisin-loaded liposomes showed that the protein interaction with phospholipids dictates the physico-chemical and biological properties of the formulation (El Jatzumi and Lafleur, 1999; Larid et al., 2003). Therefore, it seems plausible to conclude that pH and ionic strength induced protein hydrophobicity is at the basis of the

nisin/PLA interaction which is reflected in slowed and incomplete protein release.

The biological studies demonstrated that nisin was entrapped into the nano-particles in the active form indicating that the preparation conditions do not provoke irreversible protein inactivation. GAS compressed supercritical techniques work out, in fact, under mild conditions, which were already demonstrated to preserve the insulin biological activity (Caliceti et al., 2001a). Therefore, these nano-/micro-particle preparation processes seem to be more advantageous over classical techniques in order to formulate labile molecules such as proteins and oligonucleotides.

Nisin-loaded PLA nano-particles produced by GAS precipitation displayed a prolonged antibacterial activity, which was in good agreement with the in vitro release results. Interestingly, the antibacterial activity was maintained throughout 1000 h, though the slow and incomplete protein release at pH 7.4 observed in vitro. The plain PLA particles (protein unloaded nanoparticles) did not show any antibacterial activity while free nisin in solution displayed biological activity throughout 150 h. To note that the short-time activity of nisin in solution was not ascribable to the nisin withdrawn for the CFU analysis. Indeed, high amount of nisin was used in the biological assay and less than 5% of incubated nisin was withdrawn during the assay. However, the literature reports that the short-time activity of nisin can be attributed by the protein absorption by the bacterial cells, partial protein degradation or interaction with components of the release medium components.

Nisin-loaded nano-particles showed a weaker antibacterial activity within the first 3 h incubation as compared to free nisin samples. This can be due to a delay time in protein release from the polymeric matrices. Throughout the time, degraded or cell taken up nisin is replaced by active nisin freshly released by the particles. These results indicate that a formulation which provides for the protein stabilization and slow release is necessary to yield a long lasting efficient antibacterial activity while nisin solutions display very poor antimicrobial efficiency.

5. Conclusions

The results reported in the present study demonstrate that GAS precipitation techniques can be successfully applied for preparation of high quality polymeric particulate formulations. Actually, these techniques are very flexible and proper operative processes can be set up according to the physico-chemical properties of the processed materials in order to obtain nano-particles with low dispersity, high drug loading and high reproducibility. Furthermore, the mild operative GAS precipitation conditions make this technique suitable in the formulation of fragile biotechnological products such as proteins and oligonucleotides.

The nisin-loaded nano-particles are a typical example of the great potential of this technique in protein formulation. Nisin-loaded polymeric nano-particles fabricated by GAS precipitation technique was found to allow for long lasting antimicrobial activity. Indeed, this formulation provides for slow protein release and protein stabilization, which yield an efficient antimicrobial system useful in food and pharmaceutical preservation. However, environmental induced protein structure modifications as well as hydrophobic/hydrophilic variations were found to affect the protein/polymer interaction. Therefore, changes in release conditions can reflect in dramatic alteration of the protein release rate as well as the amount of released protein. This is a relevant point in formulation of polymeric systems for delivery of molecules characterised by high molecular complexity such as proteins and oligonucleotides.

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