Pharm Res (2011) 28:309–321
DOI 10.1007/s11095-010-0248-x

RESEARCH PAPER

High Loading of Gentamicin in Bioadhesive PVM/MA Nanostructured Microparticles Using Compressed Carbon-Dioxide

Elisa Elizondo • Santiago Sala • Edurne Imbuluzqueta • David González • María J. Blanco-Prieto • Carlos Gamazo • Nora Ventosa • Jaume Veciana

Received: 6 April 2010 / Accepted: 13 August 2010 / Published online: 2 December 2010

ABSTRACT

Purpose To investigate, for the first time, the viability of compressed antisolvent methodologies for the preparation of drug-loaded particles of the biodegradable and bioadhesive polymer poly (methyl vinyl ether-co-maleic anhydride) (PVM/MA), utilizing gentamicin (Gm) as a model drug.

Methods Precipitation with a Compressed Antisolvent (PCA) method was used for the preparation of PVM/MA particles loaded with gentamicin. Before encapsulation, gentamicin was modified into a hydrophobic complex, GmAOT, by exchanging its sulphate ions with an anionic surfactant. GmAOT:PVM/MA composites were fully characterized in terms of size, morphology, composition, drug distribution, phase composition, *in vitro* activity and drug release.

Results Homogeneous nanostructured microparticles of PVM/MA loaded with high and uniformly distributed quantities of GmAOT were obtained by PCA. The drug loading factors could be tuned at will, improving up to ten times the loadings obtained by other precipitation techniques. Gentamicin retained its bioactivity after being processed, and, according to its release profiles, after an initial burst it experienced a sustained release over 30 days.

Conclusions Compressed antisolvent methods are suitable technologies for the one-step preparation of highly loaded nanostructured PVM/MA matrices with promising application in the delivery of low bioavailable drugs.

KEY WORDS Gantrez AN · gentamicin · microparticles · PVM/MA · supercritical fluids

Electronic Supplementary Material The online version of this article (doi:10.1007/s11095-010-0248-x) contains supplementary material, which is available to authorized users.

E. Elizondo · S. Sala · N. Ventosa (✉) · J. Veciana (✉)
Department of Molecular Nanoscience and Organic Materials
Institut de Ciència de Materials de Barcelona (ICMAB-CSIC)
Bellaterra 08193, Spain
e-mail: ventosa@icmab.es
e-mail: vecianaj@icmab.es

E. Elizondo · S. Sala · N. Ventosa · J. Veciana
CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN)
Bellaterra, Spain

E. Imbuluzqueta · D. González · M. J. Blanco-Prieto
Department of Pharmacy and Pharmaceutical Technology
University of Navarra
Pamplona 31080, Spain

C. Gamazo
Department of Microbiology University of Navarra
Pamplona 31080, Spain

INTRODUCTION

Over the past few decades, the need of meliorating the pharmacological properties of the existing compounds more than the synthesis of new ones has aroused an increasing interest in the development of new systems for the controlled delivery of drugs. The controlled release of active compounds to the specific site of action at the therapeutically optimal rate and dose regimen has been a major goal in designing such systems. In particular, the use of micro- and nanoparticles composed by a biologically active compound and a biodegradable polymer, acting as a carrier, has been thoroughly reported during the last few decades (1–4). Both natural and synthetic polymers can be used for this purpose. Chitosan and several poly(esters), like poly(lactic acid) (PLA), poly (glycolic acid) (PGA) and poly(lactic-co-glycolic acid) (PLGA), are examples of the most studied ones (5,6).

A wide range of clinically approved pharmaceutical products takes advantage of biodegradable polymers to control the rate of drug release within the body (7,8); however, the preparation methodologies of most of these systems rely on high-temperature processing, like spray drying, or organic-solvent-based methods to incorporate the drug into the polymeric phase. High temperatures cannot be applied to temperature labile active compounds, and the formation of interfaces between organic and aqueous phases in emulsion solvent evaporation methods turns the solvent extraction step into a hard issue. Moreover, the high stirring efficiency needed in the emulsion formation step makes the scaling-up of these methodologies even more difficult.

Over the past few decades, compressed fluid (CF)-based precipitation processes have been developed as new and promising alternatives for the production of micro- and nanoparticulate materials (9–14). More recently, the main advantages of these methods—such as the small traces of organic solvent in the precipitates, lower temperature, more uniform products, less operational steps and easy scale-up—have promoted their application in the drug delivery field, focusing special attention on the processing of biodegradable polymers and drug/polymer composites (15–18). Nevertheless, the great majority of these studies has been done using poly(esters) such as PLA and PLGA as the drug carriers (19,20). Ergo, new biodegradable polymers should be investigated for the full exploitation of CF-based technologies to the preparation of drug-loaded polymeric particles.

Poly (methyl vinyl ether-*co*-maleic anhydride) (PVM/MA) is a biodegradable polymer with great potential for oral drug delivery, which is the preferred route of drug administration due to its association with patient convenience and lower costs. PVM/MA not only protects the drug from degradation but also, due to its well-studied bioadhesive properties when formulated as particles (21), interacts with the gut mucosa favoring the absorption and, therefore, the bioavailability of the carried drug. Really promising results have been reported in the use of PVM/MA nanoparticles as drug and antigen carriers (22,23); actually, poly anhydride nanoparticles are currently licensed in the UK for oral drug delivery and have proven adjuvant capacities by oral and parenteral routes (24,25). However, to our knowledge, only multi-step solvent displacement methods have been used to prepare this kind of materials.

In this work, we show, for the first time, the suitability of CF-based precipitation techniques for the production of drug:PVM/MA nanoparticulate composites, utilizing gentamicin (Gm), a widely used antibiotic, as a model drug.

Gentamicin sulphate (GS) is one of the most potent antibiotics against a wide variety of bacterial infections; however, its therapeutic efficiency is low mainly due to its high hydrophilic nature, and, consequently, high doses and frequent administrations that are limited to injection and topical dosage forms are needed to have sufficient concentration of GS at the site of infection. These excessive administrations reduce the quality of life of the patients and can induce undesirable side effects such as nephrotoxicity and ototoxicity. Therefore, there is a large interest in developing new gentamicin-based delivery systems for the efficient treatment of different kind of infections (26–29).

In this work, we have used a CF-based methodology for the preparation of the gentamicin-loaded PVM/MA particles. Prior to composite formation, the antibiotic GS, a highly hydrophilic aminoglicoside, was turned into a more lipophilic substance, GmAOT, by exchanging its sulphate ions with the anionic surfactant bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT), through the hydrophobic ion-pairing (HIP) process (30). Apart from an exhaustive physico-chemical characterization of the prepared composites, *in vitro* activity studies against the model bacteria *Brucella* were performed in order to ensure that the precipitation conditions did not affect the bioactivity of the antibiotic. *Brucella*, a BSL-3 pathogen, is recognized as facultative intracellular pathogen (31), emphasizing that it may have an extreme preference for the intracellular environment despite its ability to live outside host cells. This special feature makes it a very good model for studying new chemotherapeutic products.

MATERIALS AND METHODS

Materials

Poly (methyl vinyl ether-co-maleic anhydride) (PVM/MA), *Gantrez® AN 119* (MW 200000), was kindly given by ISP International Corp. (Sant Joan Despí, Spain). Gentamicin sulphate was purchased from Molekula (Dorset, UK). Bis (2-ethylhexyl) sulfosuccinate sodium salt (AOT) was supplied by Sigma (Tres Cantos, Spain). Carbon dioxide (purity >99.9%) was obtained from Carburos Metálicos S.A (Barcelona, Spain). Acetone (ROMIL_SpS^{TM}, purity >99.8%) was supplied by Teknokroma (Sant Cugat del Vallès, Spain). All the water used was pretreated with the Milli-Q Advantage A10 water purification system (Millipore Ibérica, Madrid, Spain).

Preparation of the Gentamicin-AOT (GmAOT) Ionic Complex

The ionic complex of gentamicin (Gm) with the anionic surfactant bis(2-ethylhexyl) sulfosuccinate (AOT), GmAOT, was prepared as a waxy solid by the hydrophobic ion-pairing (HIP) method as described elsewhere (30). Following this process, 5 mol of bis(2-ethylhexyl) sulfosuccinate sodium salt (NaAOT) were used

for the stoichiometric complexation of the 5 ionizable amino groups of gentamicin and, therefore, the replacement of the sulphate counter ions, yielding an ionic complex with a Gm:AOT ratio of 1:5. In brief, 800 mL of a solution of NaAOT in dichloromethane (12.55 mg/mL) were added to an equal volume of a buffered aqueous solution (10 mM sodium acetate, 10 mM KCl, 10 mM $CaCl_2$, pH 5.0) of GS (4 mg/mL) and stirred vigorously for 3 h. The phases were separated by centrifugation (5,000 rev./min, 5 min), and the ionic complex was recovered from the organic phase by evaporation and dried under vacuum for 15 min. The composition and molecular structure of GmAOT were confirmed by infrared (IR) spectroscopy, MALDI-TOF/MS and elemental analysis (see Supplementary Material).

Preparation of Particulate GmAOT:PVM/MA

GmAOT:PVM/MA Composites by Compressed Fluids

The antibiotic:polymer composites (C_i) were prepared by the compressed fluid-based technology called Precipitation with a Compressed Antisolvent (PCA) (32). The experiments were carried out using an experimental set-up already described before (33). The PCA-apparatus is schematized in Fig. 1. The experiments were carried out following the subsequent steps: first, the precipitation chamber, R, (300 mL) was filled with CO_2 and allowed to equilibrate to the operating pressure and temperature (10 MPa, 25°C). For achieving the desired molar fraction of CO_2, $x_{CO2}=0.95$, the back pressure valve, BP, was opened, and CO_2 and the organic solvent, acetone, were simultaneously pumped into the vessel with a volumetric CO_2:solvent ratio of 18:1 mL/min. After 20 min, the injection of pure organic solvent was stopped, and 10 mL of the solution of GmAOT and PVM/MA in acetone (0.14 g/mL) were sprayed through a hollow cone nozzle, N, (diameter = 100 μm), at the same rate of the pure solvent, into the current of CO_2. The antisolvent effect of compressed CO_2 (cCO_2) over the sprayed solution caused the precipitation of the composites. The solid particles were collected over a sintered metal filter covered with a PTFE membrane, F0, placed inside the autoclave. The removal of the possible residual solvent in the final particulate material was done by a current of CO_2 at 36.67 mL/min and 10 MPa during 1 h. The dried particulate solids were collected from the filter after depressurizing the precipitation chamber.

Physical Mixtures of GmAOT and PVM/MA

Physical mixtures (PM_i) of GmAOT and PVM/MA with the same drug:polymer ratios as the composites obtained by PCA were prepared for comparison purposes. Such particulates were prepared by grinding the desired quantities of GmAOT and PVM/MA in a mortar until a homogenous mixture of both components was achieved.

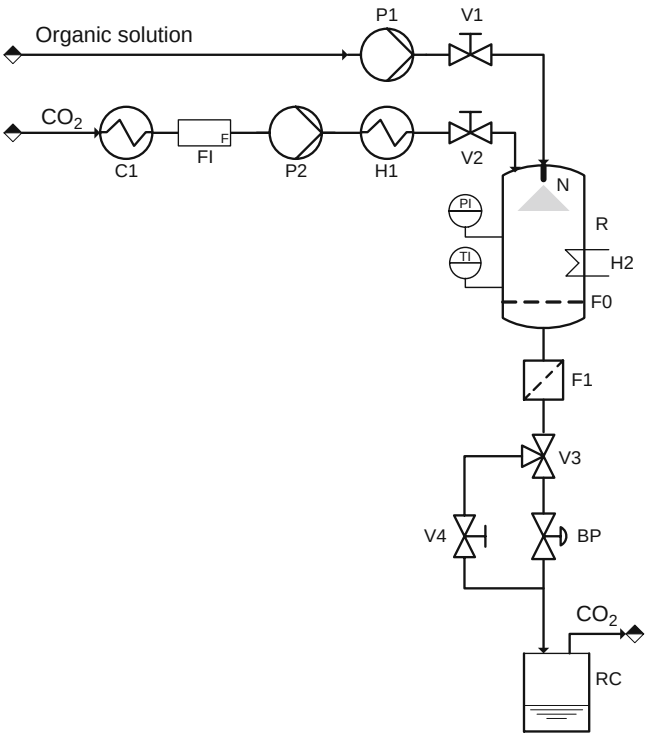

Fig. 1 Scheme of the equipment used for PCA experiments. *C*: cooler; *P*: pump; *H*: heater; *FI*: mass flow meter; *V*: valve; *BP*: back pressure valve; *F*: filter; *R*: reactor; *N*: nozzle; *RC*: recycling collector; *PI*: pressure indicator; *TI*: temperature indicator.

Physico-chemical Characterization of the Precipitates

Morphology and Gentamicin Distribution

The surface morphology of the gentamicin-loaded polymeric matrices obtained by the PCA method, C_i, was studied using a field emission scanning electron microscope (Quanta 200 FEG-ESEM, FEI, Eindhoven, The Netherlands). The samples were prepared by direct deposition of the powders onto a carbon tape placed on the surface of an aluminium stub. Prior to analysis, the samples were coated with gold for 4 min using a sputter coater (K550x, Emitech, Ashford, UK).

The gentamicin distribution into the composites and the physical mixtures was studied by Energy Dispersive X-ray (EDX) microanalysis. The elements mapping was done using the Quanta 200 FEG-ESEM (FEI, Eindhoven, The Netherlands) equipped with an EDX system for chemical analysis (EDAX, Tilburg, The Netherlands) and using the EDAX Genesis software. For the EDX study, the samples

were coated with carbon by a K550 coater with a K250 carbon coating attachment (Emitech, Ashford, UK).

The quantitative analysis of the gentacimicin distribution was performed by analysing the spectra associated to the microanalysis maps. In an EDX map, each point has an elemental analysis spectrum associated. However, the signal in the case of a light element like S is too weak, and, therefore, the selection of areas instead of points is recommended. The spectrum corresponding to each area is the average of the spectra of the points inside it. Different areas were chosen along the investigated particles. The analysis of the signal of sulphur (S), which differentiates the antibiotic from the polymer, gave quantitative information about the spatial disposition of the antibiotic in the particle.

Particle Size Distribution

The particle size distribution of the precipitates was measured by Light Scattering (LS) using a particle size analyzer (Mastersizer 2000, Malvern Instruments, UK). Prior to analysis, 3 mg of each sample were dispersed into 10 mL of deionized water and sonicated for 150 s (Ultrasons-H, Selecta, Abrera, Spain).

Drug:Polymer Chemical Interactions

In order to study possible drug:polymer chemical interactions, Fourier Transform Infrared (FTIR) spectroscopy was performed. The analyse were carried out in a Spectrum One (PERKIN ELMER, USA) spectrometer attached to an attenuated total reflectance (ATR) accessory (UATR accessory, PERKIN ELMER, USA). A small amount of sample, either the pure polymer, the pure ion paired gentamicin, GmAOT, or the different GmAOT:PVM/MA composites prepared by PCA, was directly placed on the diamond disk and scanned over the range from 4,000 to 650 cm^{-1} wave numbers at a resolution of 1 cm^{-1}.

Phase Composition

Phase transitions suffered by the pure components, the physical mixtures and the composites prepared by PCA when heating the samples from 30°C to 230°C at a heating rate of 10°C/min were analyzed using a differential scanning calorimeter (Model 822e, Mettler-Toledo, Spain). Temperature calibrations were made using indium as standard. One mg of each sample was sealed into an aluminium pan and heated under a nitrogen purge at the described conditions.

Crystallinity

The degree of crystallinity of the GmAOT inside the polymeric matrices was studied by X-ray microdiffraction using a D8 Advance X-ray diffractometer with a bidimensional detector GADDS (General Area Detector Diffraction System), (Bruker-AXS, Karlsruhe, Germany). A conventional Cu X-ray source and a 0.5 mm collimator were used. θ–2θ measurements were performed from 2.5° to 37.5° in 2θ during 900 s.

Entrapment Efficiency and Drug Loading

The study of the drug content in the different GmAOT:PVM/MA composites was performed by nuclear magnetic resonance with a BRUKER DPX-250 MHz spectrometer (BRUKER Española S.A, Barcelona, Spain). The ^{1}H NMR spectra were performed at ambient temperature using acetone-d_6 as solvent (15 mg/800 μL). The spectra analysis for drug quantification was carried out with the Top Spin software (Bruker). The integration of the appropriate non-overlapped signals of the polymer and the drug in the ^{1}H NMR spectra of the composite materials allowed us to obtain the ratio of GmAOT:PVM/MA in each composite (Fig. 2). The signals selected for quantification correspond to 5 protons in the case of the polymer (3 protons of the methoxy group and 2 protons of the cyclic anhydride; δ(ppm): 3.24–3.9) and 60 protons for the antibiotic complex GmAOT (5 molecules × 12 protons of the methyl groups of AOT; δ(ppm): 0.7–1.1) (see molecular structures in Fig. 3). The NMR experiments were performed in triplicate. Drug incorporation was expressed both as drug loading (w/w) and entrapment efficiency (%) represented by Eqs. 1 and 2, respectively, as

$$Drug\ loading\ (w/w) = \frac{Mass\ of\ drug\ in\ composites (in\ \mu g)}{Mass\ of\ composites (in\ mg)} \qquad (1)$$

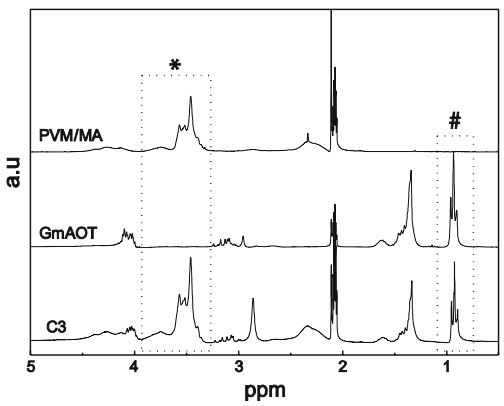

Fig. 2 ^{1}H NMR spectra of pure PVM/MA, pure GmAOT and composite C3. * Selected signal for polymer quantification. # Selected signal for antibiotic quantification.

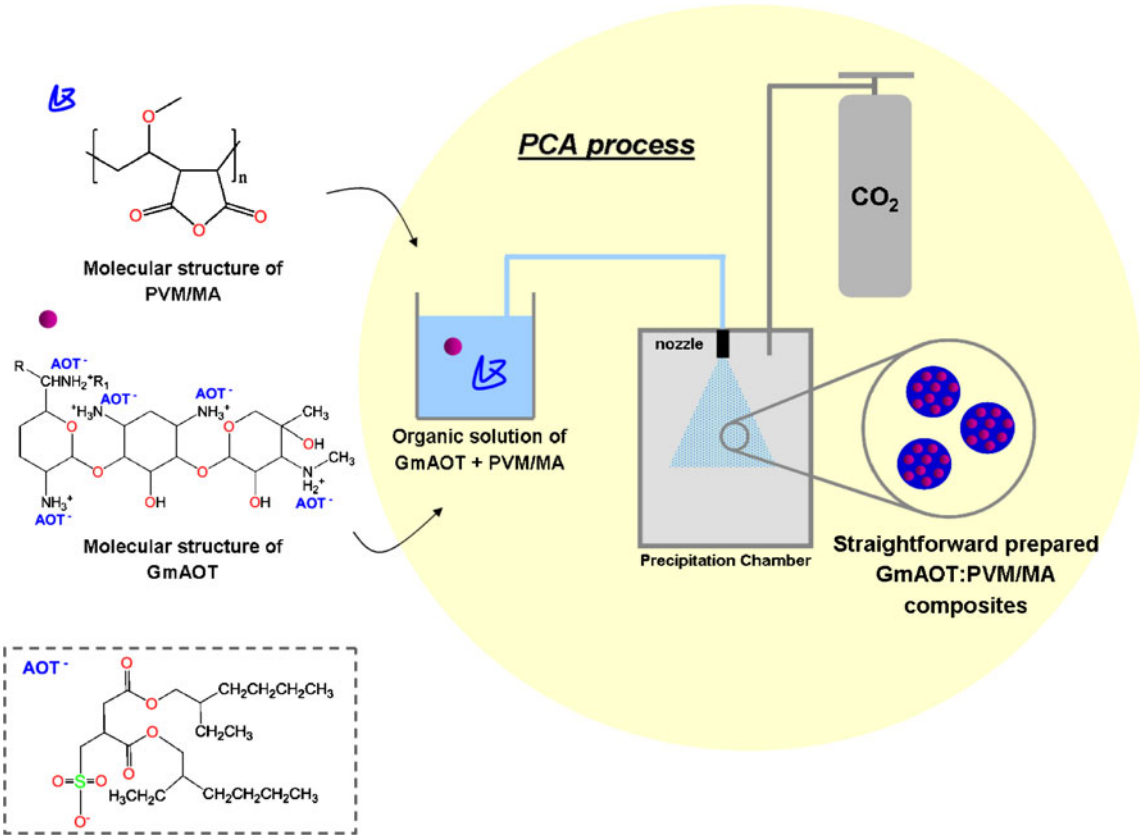

Fig. 3 Molecular structures of GmAOT and PVM/MA. Schematic representation of the PCA process for the one-step preparation of GmAOT:PVM/MA composites.

$$\textit{Entrapment efficiency}\,(\%) = \frac{\textit{Mass of drug in composites}}{\textit{Initial mass of drug}} \times 100 \qquad (2)$$

Biological Activity Studies

The reference isolate *B. melitensis* strain 16 M (ATCC 23456) was chosen for analysis. Inhibitory (MIC) and bactericidal (MBC) activity were determined by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines using cation-adjusted Mueller-Hinton broth (DIFCO BD, Franklin Lakes, USA). The MICs against *Escherichia coli* (ATCC 25922) were determined as quality control, obtaining values within the acceptable range. Briefly, 100 µL of the GmAOT complex and each GmAOT:PVM/MA composite were double-serially diluted in Microplates (TPP, Trasadingen, Switzerland). A 100-µL aliquot of organism suspension of approximately 10^6 CFU/mL was added to each well, which resulted in a starting inoculum of 5×10^5 CFU/mL. After incubation at 37°C for 48 h, MICs were defined as the lowest concentration of drug that resulted in no visible bacterial growth. Subsequently, 20 µL were removed from each well and plated on Trypticase soy agar (Biomerieux, Marcy l'Etoile, France) for MBCs determination after 5 days of incubation at 37°C. MBCs were defined as the minimum concentration of formulation that yielded ≥99.9% killing of bacteria.

In Vitro Release of GmAOT from Composites

GmAOT release profile from the carriers was measured at pH 7.4 with 0.02% (w/v) sodium azide as a bacteriostatic agent. It was confirmed that this bacteriostatic agent did not affect the release of gentamicin from the polymeric particles (data not shown). Three mg of the composites were dispersed in 1.5 mL of PBS and incubated at 37°C under orbital shaking in a rotatory plate (FALC F200, Falc intruments,Treviglio, Italy) for 4 weeks. At regular intervals, the tubes were centrifuged (21,000 g, 15 min), and from each tube supernatant was collected and replaced with fresh release medium. The collected supernatant solution was fluorometrically assayed after the derivation of Gm with o-phthalaldehyde as previously described (34). The release experiments were performed in triplicate for all the compositions. The results

were shown as the percentage of antibiotic released with regard to the amount of GmAOT encapsulation.

RESULTS

Preparation of GmAOT:PVM/MA Composites by PCA

Precipitation with a Compressed Antisolvent (PCA) process was used for the preparation of gentamicin-loaded PVM/MA matrices. By this process, a liquid solution is sprayed though a nozzle into the compressed antisolvent, which rapidly diffuses into the sprayed solution droplets, causing the precipitation of the solute (Fig. 3). Since gentamicin sulphate, the commonly used commercial form of gentamicin, has a low solubility in organic solvents, preventing its processing, the lipophilicity of the antibiotic was modified by exchanging the sulphate anion by a more hydrophobic one like the anionic surfactant bis(2-ethylhexyl) sulfosuccinate (AOT). This ion exchange was made by the hydrophobic ion pairing (HIP) method described in "Preparation of the Gentamicin-AOT (GmAOT) Ionic Complex," yielding the ionic complex GmAOT, which contains five surfactant molecules for each molecule of gentamicin. Qualitative solubility studies of the GmAOT complex and the PVM/MA polymer were performed using a high pressure variable volume cell in order to study the behaviour of compressed CO_2 (cCO_2) over organic solutions of these compounds. It was observed that for both components cCO_2 has a strong antisolvent character when the drug or the polymer are dissolved in acetone. This antisolvent effect of cCO_2 over organic solutions of both components permits the use of compressed antisolvent precipitation technologies for the co-precipitation of GmAOT:PVM/MA composites. Following the procedure described in the experimental section, five composites comprising different ratios of GmAOT and PVM/MA were prepared using the PCA conditions detailed in Table I (35). Different quantities of the ionic complex GmAOT and the polymer PVM/MA were dissolved in acetone and co-sprayed into compressed CO_2. The rapid diffusion of the cCO_2 into the sprayed solution droplets and its antisolvent effect yielded the co-precipitation of both components in the form of particulate composites which were fully characterised. The data corresponding to their composition and particle size are summarized in Table II.

Morphology and Particle Size of GmAOT:PVM/MA Composites

The scanning electron microscopy images, Fig. 4, reveal that homogeneous nanostructured composites are obtained from PCA precipitation. The analysis of a representative number (200) of particles from different SEM images of each composite gave a particle size ranging from 931 to 37 nm that depends on the used drug:polymer ratio (see Table II). Thus, a decrease in nanoparticles' size and their sphericity is observed upon augmenting the amount of GmAOT in the polymeric matrices. On the other hand, such nanoparticles appear coalesced in the form of micro-aggregates, increasing the coalescence with a higher drug content in the composites. This aggregation could be attributed to the waxy nature of the anionic surfactant AOT.

Particle size distribution of the micro-aggregates was measured by light scattering. For the particle size analysis, the polymeric composites were suspended in water and then sonicated to cause their dispersion. The results shown in Table II reveal that even though the composites are formed by particles with a primary particle size in the nanoscopic range, these particles form micro-aggregates with a medium size (D50) between 10 and 20 μm.

Solid State Characterization of the GmAOT:PVM/MA Composites

Drug:Polymer Chemical Interactions by FTIR Analysis

The FTIR spectra of the pure polymer, pure antibiotic and some of the composites are depicted in Fig. 5a. The spectrum for the pure PVM/MA shows the typical stretching bands of the anhydride group (C=O: 1,855 cm^{-1}, 1,772 cm^{-1}; C–O–C: 918 cm^{-1}) and those

Table I PCA Experiments Performed at 10 MPa, 25°C and $x_{CO2} = 0.95$ Using Acetone as Solvent and CO_2 as Compressed Antisolvent

Experiment	Composite	Sprayed solution			Precipitation yield (%)
		GmAOT:PVM/MA (w:w)	[GmAOT] (g/mL)	[PVM/MA] (g/mL)	
1	C1	0.09:1	0.01	0.13	87
2	C2	0.19:1	0.02	0.12	95
3	C3	0.37:1	0.04	0.1	100
4	C4	0.67:1	0.06	0.08	100
5	C5	1:1	0.07	0.07	94

Table II Composition and Particle Size of the GmAOT:PVM/MA Composites Prepared by PCA

Composite	Composition		Particle size			
	Drug loading (μg/mg)	Entrapment efficiency (%)	SEM (nm)[a]	Light scattering (μm)[b]		
				D[*v*,0.1]	D[*v*,0.5]	D[*v*,0.9]
C1	66 (±1)	71.8 (±0.1)	437–931	1.86	19.83	52.07
C2	145 (±10)	85.9 (±0.1)	292–448	3.14	19.69	51.78
C3	239 (±4)	88.9 (±0.1)	187–265	3.78	11.44	37.96
C4	392 (±4)	98.0 (±0.1)	76–110	2.93	10.42	34.98
C5	487 (±2)	91.6 (±0.1)	37–51	4.54	15.81	37.90

[a] Range of particle sizes measured by scanning electron microscopy, [b] Volumetric particle size distributions, measured by light scattering technique, are described by D[v,0.1], D[v,0.5] and D[v,0.9], which are the particle diameters (μm) under which there are 10%, 50% and 90% of the total volume of the sample, respectively. D[v,0.5] is the volume median particle diameter

corresponding to the methylether group (OCH_3: 1,220 cm^{-1}, 1,088 cm^{-1}). The most intense bands in the spectrum of GmAOT correspond to the functional groups of the surfactant AOT. Thus, bands between 3,000 and 2,840 cm^{-1} are characteristic of the alkyl chains of the surfactant, while those at 1,734, 1,200, 1,158 cm^{-1} are attributed to the ester group and the band at 1,034 cm^{-1} corresponds to the SO_3^- group. The FTIR spectra corresponding to the composites are just the juxtaposition of those of the pure drug and polymer. It can be observed that the intensity of the bands corresponding to the antibiotic, almost masked by those of the polymer in the case of the less-loaded composite, increase by augmenting the quantity of GmAOT in the composite. Neither appreciable shifts, nor the appearance or disappearance of bands are observed in the composites spectra, indicating that there are no significant interactions between both components in the solid state.

Crystalline Degree by Thermal Analysis and X-ray Diffraction

In order to study the phase composition of the composites, DSC and X-ray measurements were conducted. DSC thermograms of PVM/MA, GmAOT, the prepared composites (C1–C5) and their physical mixtures obtained by grinding the same GmAOT:PVM/MA ratios (PM1–PM5) are presented in Fig. 5c. The pure polymer presents a small endothermic peak at 157°C assigned to its glass transition temperature (Tg), while the thermogram corresponding to the antibiotic shows an endothermic melting peak at 212°C. The latter peak is present in all the physical mixture samples and the three

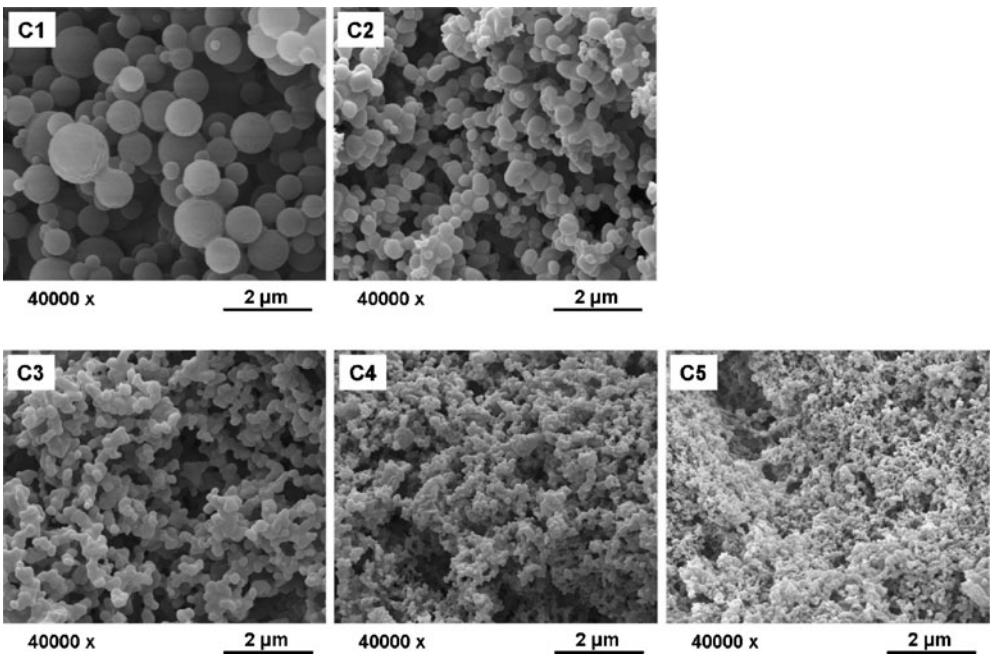

Fig. 4 SEM micrographs corresponding to the five different GmAOT:PVM/MA composites.

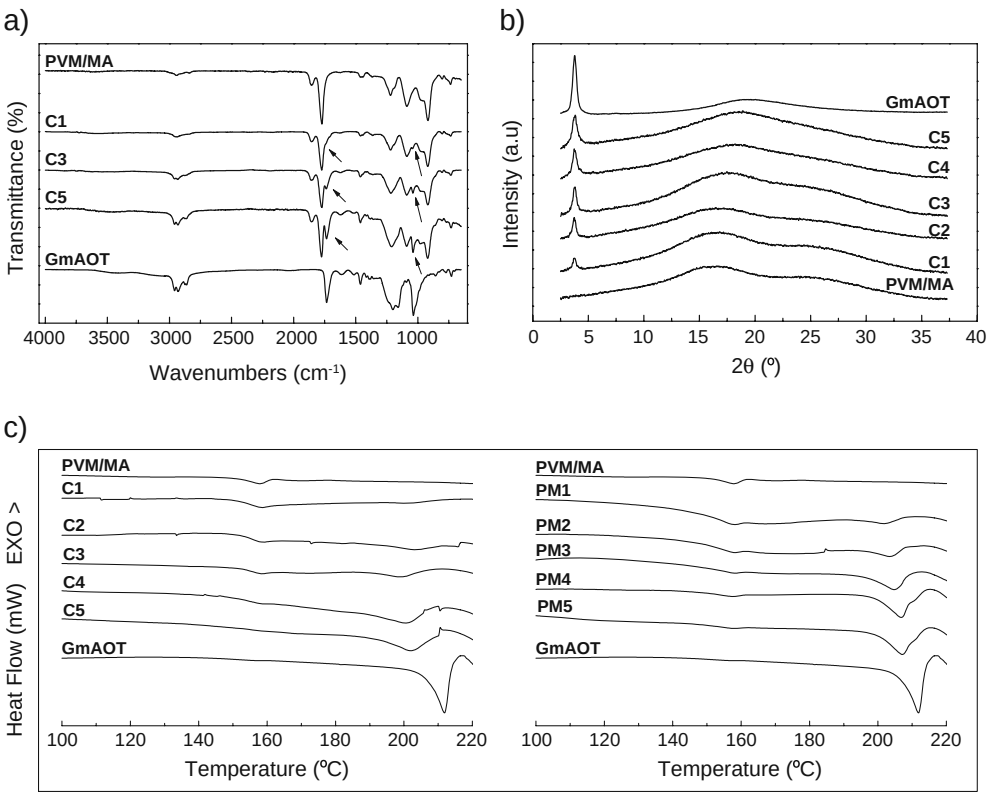

Fig. 5 FTIR spectra (**a**), X-ray diffractograms (**b**) and DSC thermograms (**c**) of PVM/MA, GmAOT and GmAOT:PVM/MA composites. In (**c**) both, the different composites (*left*) and the physical mixtures (*right*) are depicted.

more-loaded composites; however, its absence is observed for the composites with lower antibiotic concentration (C1 and C2). Although this could indicate that GmAOT becomes amorphous inside the polymeric matrix for these compositions, X-ray measurements contradict this assumption. Indeed, the X-ray diffractograms, depicted in Fig. 5b, show that apart from the wide signal corresponding to the amorphous nature of the polymer, all the composites show a peak at 3.76° in 2θ corresponding to the antibiotic, revealing, therefore, that the presence of a long-range order of GmAOT is maintained after the co-precipitation with the polymer by PCA for every composition. DSC shows that the melting peak for the GmAOT in both physical mixtures and composites is broadened and occurs at lower temperatures compared to the one of the pure antibiotic. These effects, which are more evident for the composites than for the physical mixtures (PM), could be related to a polymer/antibiotic solid state interaction induced by heating as previously reported by Tantishaiyakul *et al.* for piroxicam and PVP K-17 (36). This behaviour observed by DSC was corroborated by hot stage microscopy images (see Supplementary Material).

Composition of the GmAOT:PVM/MA Composites

Entrapment Efficiency and Drug Loading

The composition of the composites in terms of drug loading and entrapment efficiency is listed in Table II. ^{1}H NMR was used as a quantitative tool to determine the ratio between GmAOT and PVM/MA in each composite. It was observed that the ratios between the polymer and the drug in the precipitates were practically the same as the ones in the injected solutions and that the entrapment efficiencies were more than 85% for the majority of compositions. The antibiotic loadings obtained in the present work could be changed in almost one order of magnitude, ranging from 65 to almost 500 μg/mg, just by increasing the amount of antibiotic in the injected solution.

Gentamicin Distribution in the Composites

In order to study the distribution of gentamicin in the polymeric matrices, EDX microanalysis was performed. This technique reveals which elements are present in a solid sample but also provides information about the spatial disposition of these elements in the sample. GmAOT and PVM/MA are formed by carbon (C), hydrogen (H) and oxygen (O) atoms, whereas GmAOT also contains nitrogen (N) and sulphur (S). Therefore, enough difference exists to distinguish its presence by EDX microanalysis. Due to the low intensity of the N peak and its interference with the signals coming from C and O in the spectrum, S was the element chosen for tracking the location of the drug in the co-precipitated powders. Figure 6 discloses an example of the EDX analysis done for one composite and one physical mixture of GmAOT and PVM/MA. SEM micrographs of

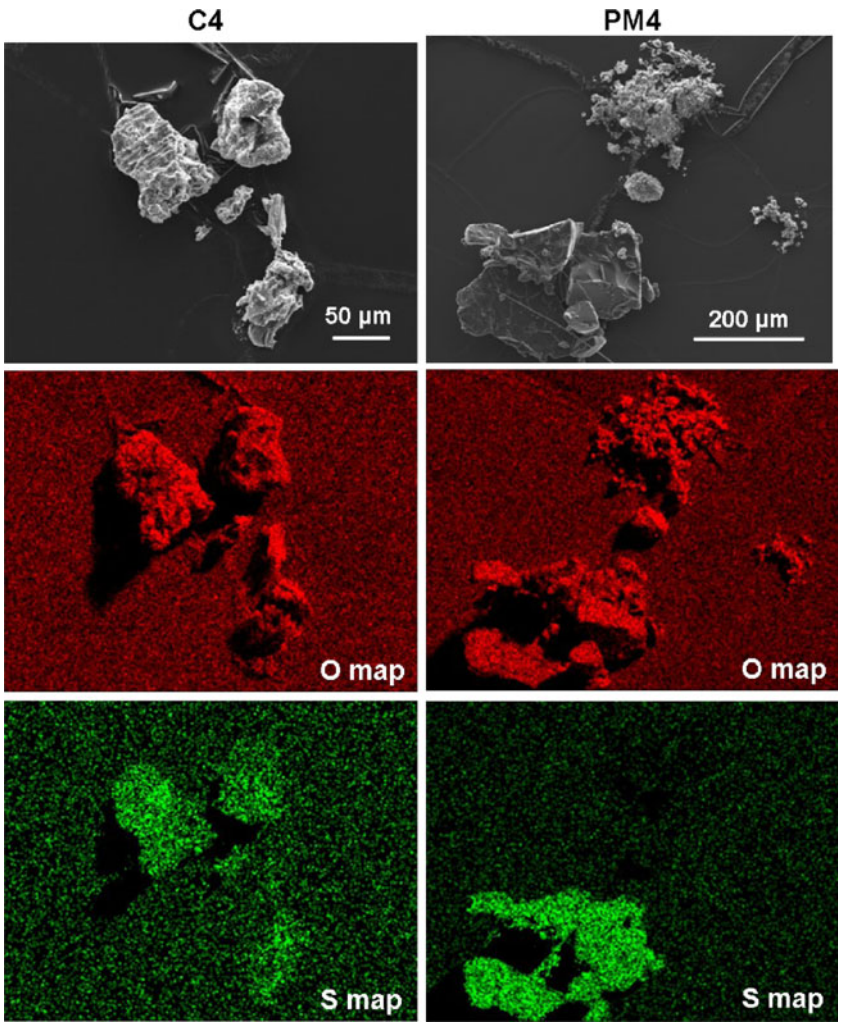

Fig. 6 EDX analysis of a composite prepared by PCA (C4) and a physical mixture (PM4) of GmAOT and PVM/MA. SEM image (*top*), oxygen map (O map) and sulphur map (S map).

the analyzed areas and the oxygen and sulphur maps for both samples revealed striking differences between them. Thus, the S map of PM4 shows that grinding antibiotic and polymer particles does not produce an intimate dispersion in which GmAOT is loaded in the polymeric matrix but a mixture of separate particles of antibiotic and polymer, with and without S atoms, respectively. On the contrary, in the case of the composite C4 prepared by PCA, there is S in all the micro-aggregates and its distribution is homogeneous inside each of them, indicating that the antibiotic is uniformly distributed in the whole polymeric matrix.

A quantitative study of the gentamicin spatial disposition in PM4 and C4 was also performed. Different areas along each particle were chosen for the quantitative analysis of the sulphur signal of their corresponding spectra. Figure 7 clearly shows that in the case of the physical mixture the particles of polymer and antibiotic are completely separated, encountering, as a result, particles with no S and particles with an identical S signal along them. On the contrary, the different selected areas both along the same particle and between different particles of the composite have comparable S signals denoting an homogeneous distribution of the antibiotic. As expected, the S signal of the composite particles is lower than that of the pure GmAOT particles present in PM4.

Inhibitory and Bactericidal Activity of GmAOT and GmAOT:PVM/MA Composites

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined for GmAOT and each GmAOT:PVM/MA composite.

In order to study the activity of the new Gm formulations, *Brucella* was used as the target pathogen. *Brucella* is considered as facultative intracellular bacteria due to its extraordinary ability to survive inside cells and high resistance that allows it to survive in the extracellular milieu (37). Gentamicin maintained its activity irrespective of the coupling to AOT or the encapsulation in PVM/MA composites. As shown in Table III, the MIC for all tested treatments was just one double-dilution above the MIC of

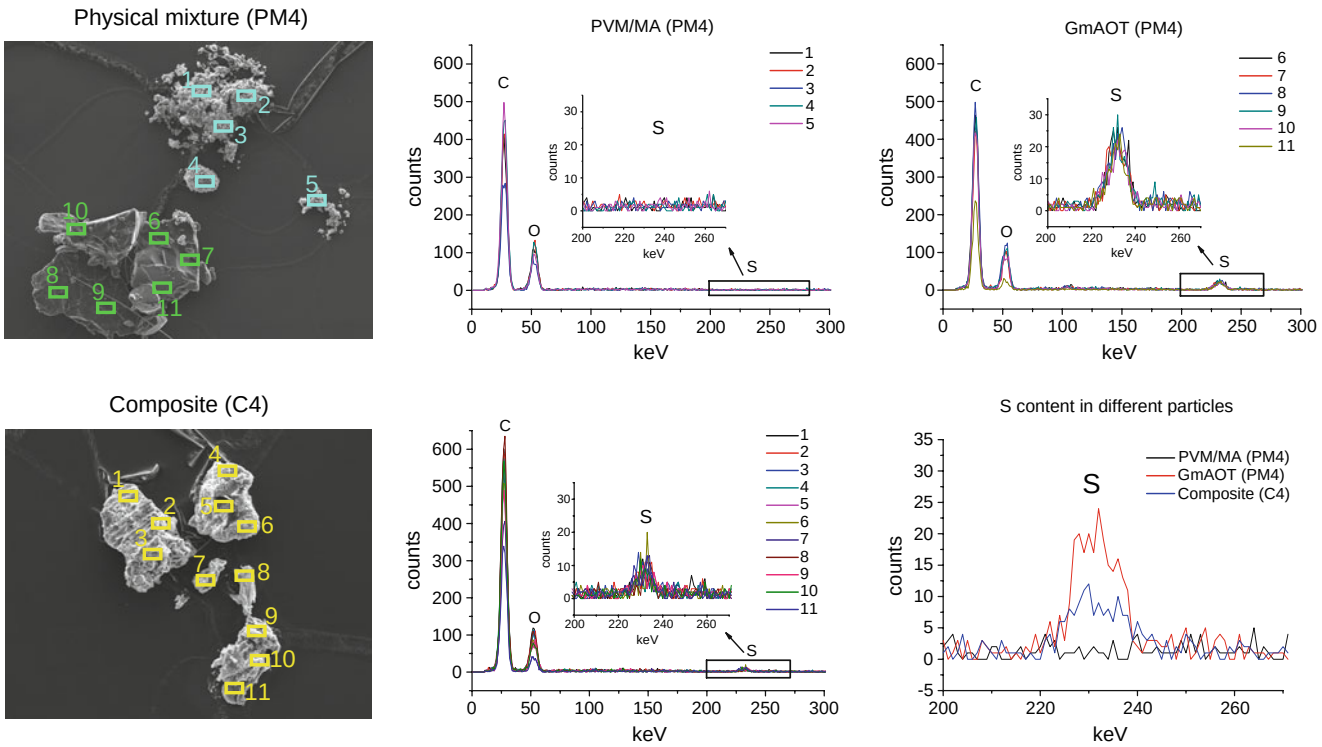

Fig. 7 EDX Microanalysis maps of physical mixture PM4 (*top*) and composite C4 (*bottom-left*) showing gentamicine spatial distribution. Top: SEM image with the areas chosen for quantitative elemental analysis of PM4 and their corresponding spectra. Bottom-left: SEM image with the areas chosen for quantitative elemental analysis of C4 and their corresponding spectra. Bottom-right: comparison of the S signals for the pure GmAOT (PM4), pure PVM/MA (PM4) and C4.

GS, our reference compound. AOT alone did not show any activity against the bacterial inoculum (above 32 μg/mL), indicating that the activities of GmAOT and GmAOT:PMV/MA composites were due to Gm. MBC values were similar to MIC, confirming that the bactericidal activity of Gm was unmodified after AOT-coupling or entrapping in PVM/MA particles.

GmAOT *In Vitro* Release Kinetics

In vitro release profile of GmAOT was evaluated only for C4 and C5 composites since they have the highest antibiotic concentration and therefore present the best characteristics for further *in vivo* application. *In vitro* release profile of GmAOT exhibited a biphasic pattern characterized by an initial burst during the first 24 h, followed by a slow sustained release until day 14 (Fig. 8). The highest burst release corresponded to the composite C5, which released about 18%, in contrast to the composite C4 that, by the same time, released 8.5% of its drug content. The initial burst corresponds either to the release of the antibiotic adsorbed onto the surface or to the diffusion of the GmAOT from the areas close to the surface of the particles. This release was higher for the composite with the higher loading of GmAOT. After 14 days, an increase in the antibiotic release rate was observed until the end of the

Table III MICs and MBCs for GmAOT:PMV/MA Composites Against *Brucella melitensis*

Treatment	GmAOT:PVM/MA (w:w)	MIC (μg/mL)	MBC (μg/mL)
GS		0.25	0.5
GmAOT		0.5	1
Composite C1	0.09:1	0.5	1
Composite C2	0.19:1	0.5	1
Composite C3	0.37:1	0.5	1
Composite C4	0.67:1	0.5	1
Composite C5	1:1	0.5	1
AOT		32	64

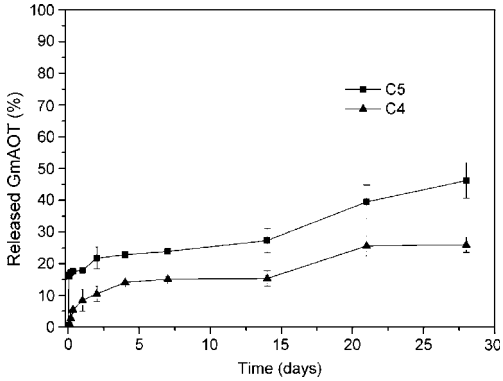

Fig. 8 Cumulative *in vitro* release profiles of GmAOT from C4 and C5 composites in PBS ($n = 3$).

study. At this point the accumulative drug release of C5 and C4 composites reached 46% and 26%, respectively.

DISCUSSION

Although much less explored than polymers like PLA or PLGA, the copolymer PVM/MA has already shown promising properties as a new material to prepare bioadhesive nanoparticles for oral drug delivery and vaccination (21–25). However, the reduced operational steps and robustness, among other advantages of the CF-based technologies, have been still unexplored for this emerging and interesting polymer. The micronization of PVM/MA by CFs and the influence of the operating parameters in the morphology and particle size of the precipitates have recently been studied by our group (38). In this work, in a further step towards prospecting possible applications for this polymer, we have probed, for the first time, the viability of compressed antisolvent methodologies for the loading of drugs, using gentamicin as a model, into the biodegradable and bioadhesive polymer PVM/MA.

By carefully selecting the appropriate PCA conditions, highly loaded PVM/MA microparticles with a medium size ranging between 10 and 20 μm were obtained. Instead of having a smooth surface, these microparticles were homogeneously nanostructured, being formed by networked nanoparticles whose size decreased from 931 to 37 nm depending on the drug:polymer ratio in the sprayed solution. This nanostructure confers them large accessible surface areas that once dispersed in aqueous media might represent a great advantage by providing a better bioadhesion due to a major contact area with the mucosa. Additionally, the nanostructured microparticles were successfully loaded with high quantities of GmAOT, the entrapment efficiencies being more than 85% for the majority of experiments. This result points out an important benefit of using PCA methodology for preparing drug-loaded polymeric matrices. The strong antisolvent effect of cCO_2, for both drug and polymer, that makes precipitation yields close to 100% allowed us to easily tune the drug: polymer composition of the resulting precipitates just by changing this ratio in the injected solution. The achieved drug loadings are, indeed, considerably higher than those reported for other polymeric micro- and nanoparticles containing gentamicin, having the composite C5 more than ten times more antibiotic (27,39). Moreover, it was proved that the antibiotic and the polymer had no chemical interactions in the solid state even if they were intimately mixed forming a solid dispersion in which the antibiotic retained its long-distance order. This good dispersion was enhanced by the co-precipitation by PCA, since, as it can be extracted from the DSC thermograms, the possible drug:polymer solid-state interactions upon heating are more pronounced for the composites than for the corresponding physical mixtures, in which GmAOT started to melt six degrees after than when loaded into the polymer by PCA. This could indicate a much better dispersion of the antibiotic into the polymeric matrix and, therefore, a more homogeneous material than the physical mixtures. This homogeneity was corroborated by EDX results which indicated that the antibiotic is uniformly distributed in the whole polymeric matrix. This uniform disposition of the drug has also been achieved by other authors for other drug/polymer composites prepared by compressed fluids-based methods (40). GmAOT release profiles of the most-loaded formulations revealed that after an initial burst release of the antibiotic located onto or near the surface of the particles, a continuous release of the antibiotic was observed for over 1 month.

Gentamicin is brucellicidal *in vitro*, but the need for parenteral administration and its poor capacity to go through cells reduce its therapeutical application (41). In this context, the current proposed formulations, which proved to retain the bactericidal action of gentamicin, might help to increase the therapeutic index of this antimicrobial in the intracellular milieu, while avoiding problems associated with administering high doses of antibiotic systemically.

CONCLUSIONS

Precipitation with a Compressed Antisolvent (PCA), using CO_2 and acetone as organic solvent, was demonstrated to be a suitable process to prepare homogenous nanostructured microparticles of GmAOT:PVM/MA composites. The resulting composites were successfully loaded with high quantities of the antibiotic, which is homogeneously distributed into the polymeric matrix. Moreover, the use of cCO_2 did not reduce the *in vitro* activity of gentamicin in the composites and permitted higher loading factors

compared to other precipitation techniques, which can be tuned at will just by using different initial ratios of the drug and the polymer. As the clinical use of gentamicin sulphate is limited to injection and topical dosage forms due to its low bioavailability, these high-loaded composites could provide a suitable oral dosage form for easier and more convenient treatments. The results described in this work open the possibility to use the same strategy for the preparation, in a single step, of nanostructured microparticles of PVM/MA loaded with other lipophilic drugs with low bioavailability and problematic oral delivery.

ACKNOWLEDGMENTS

This work was supported by the "Plan Nacional de Investigación Científica y Desarrollo e Innovación Tecnológica," Iniciativa Ingenio 2010, Programa *Consolider*, under projects EMOCIONA (CTQ2006-06333/BQU) and NANOFAR (NAN2004-09159-C04), Caja Navarra Foundation and CIBER-BBN, which is an initiative funded by the VI National R&D&i Plan 2008-2011, Inicitiva Ingenio 2010, Consolider Program, CIBER Actions and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. The authors wish to thank ISP International Corp. for supplying the biodegradable polymer, Judith Oró and Ana Esther Carrillo for the technical support in taking SEM images, Ana Crespi for her assistance in the X-ray measurements, and MATGAS personnel for the technical assessment with the high pressure equipments. E. E. thanks Consejo Superior de Investigaciones Científicas (CSIC) for her Ph. D. bursary. N. V. thanks Banco de Santander for the Chair of Knowledge and Technology Transfer Parc de Recerca UAB-Santander. E. I. thanks the Spanish "Ministerio de Ciencia e Innovación" for the fellowship Grants.

REFERENCES

1. Tabata Y, Gutta S, Langer R. Controlled delivery systems for proteins using polyanhydride microspheres. Pharm Res. 1993;10:487–96.
2. Uhrich KE, Cannizzaro SM, Langer RS, Shakesheff KM. Polymeric systems for controlled drug release. Chem Rev. 1999;99:3181–98.
3. Soppimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE. Biodegradable polymeric nanoparticles as drug delivery devices. J Control Release. 2001;70:1–20.
4. Pridgen EM, Langer R, Farokhzad OC. Biodegradable, polymeric nanoparticle delivery systems for cancer therapy. Nanomedicine. 2007;2:669–80.
5. de Campos AM, Diebold Y, Carvalho ELS, Sanchez A, Alonso MJ. Chitosan nanoparticles as new ocular drug delivery systems: *in vitro* stability, *in vivo* fate, and cellular toxicity. Pharm Res. 2004;21:803–10.
6. O'Hara P, Hickey AJ. Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: manufacture and characterization. Pharm Res. 2000;17:955–61.
7. Tracy MA. Development and scale-up of a microsphere protein delivery system. Biotechnol Prog. 1998;14:108–15.
8. Okada H. One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate. Adv Drug Deliv Rev. 1997;28:43–70.
9. Matson DW, Fulton JL, Petersen RC, Smith RD. Rapid expansion of supercritical fluid solutions: solute formation of powders, thin films and fibers. Ind Eng Chem Res. 1987;26:2298–306.
10. Gallagher PM, Coffey MP, Krukonis VJ, Klasutis N. Gas antisolvent recrystallization—new process to recrystallize compounds insoluble in supercritical fluids. ACS Symp Ser. 1989;406:334–54.
11. Bleich J, Müller BW, Wassmus W. Aerosol solvent extraction system—a new microparticle production technique. Int J Pharm. 1993;97:111–7.
12. Reverchon E. Supercritical antisolvent precipitation of micro- and nano-particles. J Supercrit Fluids. 1999;15:1–21.
13. Weidner E, Knez Z, Novak Z. PGSS (Particles from gas saturated solutions)—a new process for powder generation, Proceedings of the third International Symposium on Supercritical Fluids, France, 1994, pp. 229–234.
14. Ventosa N, Sala S, Veciana J, Llibre J, Torres J. Depressurization of an expanded organic liquid solution (DELOS): a new procedure for obtaining submicron- or micron-sized crystalling particles. Cryst Growth Des. 2001;1:299–303.
15. Bodmeier R, Wang H, Dixon DJ, Mawson S, Johnston KP. Polymeric microspheres prepared by spraying into compressed carbon-dioxide. Pharm Res. 1995;12:1211–7.
16. Yeo SD, Kiran E. Formation of polymer particles with supercritical fluids: a review. J Supercrit Fluids. 2005;34:287–308.
17. Mishima K. Biodegradable particle formation for drug and gene delivery using supercritical fluid and dense gas. Adv Drug Deliv Rev. 2008;60:411–32.
18. Reverchon E, Adami R, Cardea S, Della Porta G. Supercritical fluids processing of polymers for pharmaceutical and medical applications. J Supercrit Fluids. 2009;47:484–92.
19. Elvassore N, Bertucco A, Caliceti P. Production of protein-loaded polymeric microcapsules by compressed CO_2 in a mixed solvent. Ind Eng Chem Res. 2001;40:795–800.
20. Ghaderi R, Artursson P, Carlfors J. A new method for preparing biodegradable microparticles and entrapment of hydrocortisone in DL-PLG microparticles using supercritical fluids. Eur J Pharm Sci. 2000;10:1–9.
21. Arbós P, Campanero MA, Arangoa MA, Renedo MJ, Irache JM. Influence of the surface characteristics of PVM/MA nanoparticles on their bioadhesive properties. J Control Release. 2003;89:19–30.
22. Arbós P, Campanero MA, Arangoa MA, Irache JM. Nanoparticles with specific bioadhesive properties to circumvent the pre-systemic degradation of fluorinated pyrimidines. J Control Release. 2004;96:55–65.
23. Salman HH, Gamazo C, de Smidt PC, Russell-Jones G, Irache JM. Evaluation of bioadhesive capacity and immunoadjuvant properties of vitamin B_{12}-gantrez nanoparticles. Pharm Res. 2008;25:2859–68.
24. Salman HH, Irache JM, Gamazo C. Immunoadjuvant capacity of flagellin and mannosamine-coated poly(anhydride) nanoparticles in oral vaccination. Vaccine. 2009;27:4784–90.
25. Irache JM, Salman HH, Gómez S, Espuelas S, Gamazo C. Poly (anhydride) nanoparticles as adjuvants for mucosal vaccination. Front Biosci. 2010 (*in press*).

26. Meyer JD, Falk RF, Kelly RM, Shively JE, Withrow SJ, Dernell WS, *et al.* Preparation and *in vitro* characterization of gentamycin-impregnated biodegradable beads suitable for treatment of osteomyelitis. J Pharm Sci. 1998;87:1149–54.
27. Virto MR, Elorza B, Torrado S, Elorza MA, Frutos G. Improvement of gentamicin poly(D, L-lactic-co-glycolic acid) microspheres for treatment of osteomyelitis induced by orthopedic procedures. Biomaterials. 2007;28:877–85.
28. Lecaroz C, Gamazo C, Blanco-Prieto MJ. Nanocarriers with gentamicin to treat intracellular pathogens. J Nanosci Nanotechnol. 2006;6:3296–302.
29. Gamazo C, Prior S, Lecaroz MC, Vitas AI, Campanero MA, Pérez G, *et al.* Biodegradable gentamicin delivery systems for parenteral use for the treatment of intracellular bacterial infections. Expert Opin Drug Deliv. 2007;4:677–88.
30. Falk R, Randolph TW, Meyer JD, Kelly RM, Manning MC. Controlled release of ionic compounds from poly(L-lactide) microspheres produced by precipitation with a compressed antisolvent. J Control Release. 1997;44:77–85.
31. Moreno E, Moriyon I. Brucella melitensis: A nasty bug with hidden credentials for virulence. Proc Natl Acad Sci USA. 2002;99:1–3.
32. Dixon DJ, Johnston KP, Bodmeier RA. Polymeric materials formed by precipitation with a compressed fluid antisolvent. AIChE J. 1993;39:127–39.
33. Gimeno M, Ventosa N, Boumghar Y, Fournier J, Boucher I, Veciana J. Micronization of the chitosan derivatives D-glucosamine hydrochloride and D-glucosamine sulphate salts by dense gas antisolvent precipitation techniques. J Supercrit Fluids. 2006;38:94–102.
34. Blanco-Prieto MJ, Lecaroz C, Renedo MJ, Kunkova J, Gamazo C. *In vitro* evaluation of gentamicin released from microparticles. Int J Pharm. 2002;242:203–6.
35. Elizondo E, Ventosa N, Sala S, Veciana J, Blanco-Prieto MJ, Gamazo C, González D, Imbuluzqueta E. Composition comprising gentamicin, an anionic surfactant and a copolymer. WO Patent No 071730 A1, 11 June (2009).
36. Tantishaiyakul V, Kaewnopparat N, Ingkatawornwong S. Properties of solid dispersions of piroxicam in polyvinylpyrrolidone. Int J Pharm. 1999;181:143–51.
37. Corbel MJ. In: Young EJ, Corbel MJ, editors. Microbiology of the genus Brucella in Brucellosis: clinical and laboratory aspects. Boca Raton: CRC Press, Inc.; 1989. p. 53–72.
38. Elizondo E, Córdoba A, Sala S, Ventosa N, Veciana J. Preparation of biodegradable poly (methyl vinyl ether-*co*-maleic anhydride) nanostructured microparticles by precipitation with a compressed antisolvent. J Supercrit Fluids. 2010;53:108–114.
39. Lecaroz MC, Blanco-Prieto MJ, Campanero MA, Salman H, Gamazo C. Poly(D, L-Lactide-Coglycolide) particles containing gentamicin: pharmacokinetics and pharmacodynamics in Brucella melitensis-infected mice. Antimicrob Agents Chemother. 2007;51:1185–90.
40. Reverchon E, Antonacci A. Drug-polymer microparticles produced by supercritical assisted atomization. Biotechnol Bioeng. 2007;97:1626–37.
41. Lecároz C. Determination of gentamicin in different matrices by a new sensitive high-performance liquid chromatography-mass spectrometric method. J Antimicrob Chemother. 2006;58:557–63.