

# Production of Nanostructured Microspheres Biopolymer-Active Principle-Magnetic Nanoparticles by Supercritical Assisted Atomization

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**Abstract** Supercritical Assisted Atomization (SAA) has been applied to the production of nanostructured microspheres ampicillin-chitosan-magnetic nanoparticles (AMP-CH-NMPs). Several ampicillin/chitosan (AMP/CH) ratios with a fixed content of NMPs were processed in acid water solutions, to produce microspheres with different size, drug content and amount of NMPs. To verify the successful formation of microparticles, drug content and nanoparticle dispersion, they were characterized by SEM (Scanning Electron Microscope), EDX (Energy Dispersive X-ray), TGA (ThermoGravimetric Analysis), HPLC (High Performance Liquid Chromatography), UV-vis obtaining information on morphology, particle size distribution, nanostructure, loading of active principle in the polymeric matrix and drug release rate. Spherical microparticles were obtained, with a maximum particle size of 2  $\mu\text{m}$  and loading efficiencies up to 99%. The microspheres produced by SAA showed a controlled release of the drug over about 72 h.

**Keywords** Supercritical assisted atomization • Magnetic nanoparticles • Nanostructured microparticles • Controlled release • Targeted delivery • Ampicillin trihydrate

## 1 Introduction

Nanoparticles with Magnetic Properties (NMPs) can be used as contrast agent in magnetic resonance imaging (MRI) [1], as hyperthermia agents and as vectors for the targeted delivery of drugs; in this case they will be coprecipitated with the drug and driven to the target tissue [2]. NMPs have unique physical properties and ability to act at the cellular and molecular level of biological interactions [3].

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Encapsulation of antibiotics in nanoparticles increases the maximum tolerated dose and therapeutic index of antibiotics when compared with the free drug [4].

In this work Supercritical Assisted Atomization (SAA) has been used for the production of nanostructured microspheres biopolymer-ampicillin-NMPs for biomedical applications. The aim was to entrap the drug and the NMPs in a polymer carrier that has the role to protect the active principle and to control its release. Moreover, the encapsulation in a polymeric coating makes the NMPs biocompatible and the polymer provides a steric barrier to prevent nanoparticle agglomeration [5]. Chitosan (CH) has been selected: it is a biopolymer of great interest as a carrier because of its natural origin; ampicillin tri-hydrate (AMP) has been chosen as model drug;  $\text{Fe}_3\text{O}_4$  nanoparticles have been used as NMPs.

In literature there are some attempts at loading ampicillin in chitosan, in form of beads (850–1100  $\mu\text{m}$ ) or microgranules by coagulation of complex pastes followed by drying or in form of nanoparticles by ionic gelation method with the aid of sonication [6–8]. Nanoparticles of ampicillin trihydrate loaded in chitosan demonstrated superior antimicrobial activity with respect to plain nanoparticles and the reference, due probably to the synergistic effect of chitosan and the drug. In vitro release data showed an initial burst followed by slow sustained drug release [7].

The use of NMPs for treating infections is not well documented, on the other hand antibiotics are widely used in modern medicine [9]. Nanoparticle-based antimicrobial agents exhibit several advantages, including their inhibitory and/or killing effect on various microorganisms [10]. Hussein-Al-Ali et al. [11] prepared AMP-CH- $\text{Fe}_3\text{O}_4$  nanocomposite using a three step process: first NMPs were prepared through an iron salt coprecipitation method in an alkaline medium, followed by a chitosan coating step for the production of CH-coated NMPs, finally, the NMPs were loaded with AMP mixing AMP aqueous solution with CH-NMPs to form AMP-CH-NMP nanocomposite. The synthesized nanocomposites exhibited antibacterial and antifungal properties, as well as antimycobacterial effects.

To overcome the limitations of the traditional techniques, supercritical fluid based processes have been developed for several applications [12–14]. In particular, Supercritical Antisolvent (SAS), Supercritical Assisted Atomization (SAA), Supercritical Fluid Extraction from Emulsion (SEE) have been developed to produce micro and nanoparticles [15–25]. SAA has been successfully used for the production of co-precipitates, as nano-structured microparticles formed by a carrier in which the active principle is uniformly distributed [26–31] or as nanostructured polymeric microparticles loaded with nanoparticles [32, 33]. SAA is based on the formation of organic solvent + solid solutions in which  $\text{SC-CO}_2$  is solubilized to form an expanded liquid of reduced viscosity and surface tension. These conditions produce an improved atomization, producing controlled micro and submicro droplets that, upon drying, form the corresponding polymer + drug coprecipitated microparticles [27, 28].

In previous works, the formation of polymer microparticles loaded with NMPs [32] and the production of AMP/CH microspheres [34] using SAA technique has been studied. In this work the formation of complex composite microparticles is

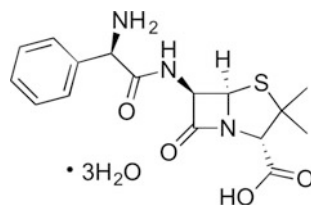
proposed, starting from a suspension in which NMPs have been finely dispersed in a solution containing CH and AMP. It is the first time that a complex mixture of three compounds is processed by SAA. The aim is to obtain coprecipitates that can be applied in the targeted delivery and allow the sustained release of the drug when it is on the site of delivery. Several ratios AMP/CH are investigated, namely 1/2, 1/4 and 1/6 wt/wt and the effect on particle size distribution and AMP controlled release are studied.

## 2 Materials and Methods

Chitosan, medium molecular weight (CH), ampicillin trihydrate (AMP, Fig. 1) and  $\text{Fe}_3\text{O}_4$  nanopowder ( $d_n < 50$  nm, >98% trace metal basis) were supplied by Sigma-Aldrich (Milan, Italy). Water (HPLC grade) with a purity of 99.5% was supplied by Sigma-Aldrich (Milan, Italy). Carbon dioxide ( $\text{CO}_2$ ; purity 99.9%) and Nitrogen ( $\text{N}_2$ ; purity 99.9%) were purchased from SON (Naples, Italy).

In order to verify the stability of the suspension before the micronization process, solutions of acidic water at 1% v/v acetic acid and CH were prepared and Tween 80 was chosen as surfactant, because of its compatibility with water and with pharmaceutical processes. Solutions at concentrations of CH of 10 and 20 mg/mL and Tween 80 at different % wt/wt surfactant/solvent were sonicated in an ultrasound bath for 10 min. Then, increasing amounts of  $\text{Fe}_3\text{O}_4$  nanoparticles (mean diameter 50 nm) were suspended in the solution and sonicated using a high-intensity ultrasonic probe at 50% amplitude for 1 min at pulse mode and their stability was monitored. SAA laboratory apparatus used is composed by two high-pressure pumps (mod. 305, Gilson, Villiers Le Bel, France) to deliver the water solution and the liquid  $\text{CO}_2$  to the saturator. The saturator is a heated high pressure packed vessel (volume: 25  $\text{cm}^3$ ) which assures a large contact surface between liquid solution and  $\text{CO}_2$ . The expanded liquid obtained in the saturator is sprayed through a nozzle into the precipitator ( $v = 3$   $\text{dm}^3$ ) that operates at atmospheric pressure. A controlled heated flow of  $\text{N}_2$  (about 1200 nL/h) is flown to the precipitator to enhance the evaporation of water from the droplets. A sintered filter at the bottom of the precipitator, with a porosity of 0.5  $\mu\text{m}$ , allows the collection of the powder and the flowing through of the gases. SAA apparatus layout and further details can be found in previous papers [24].

**Fig. 1** Molecular structure of Ampicillin trihydrate



The morphology of produced particles has been analysed by a Field Emission Scanning Electron Microscope (FESEM, mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany). Powders were dispersed on a carbon tab previously stuck to an aluminium stub (Agar Scientific, United Kingdom). Samples were coated with gold (layer thickness 150 Å) using a sputter coater (mod. 108 Å, Agar Scientific, Stansted, United Kingdom). Several SEM images were taken for each batch to verify the powder uniformity.

Particle size (PS) and particle size distribution (PSD) were measured from FESEM images using the Sigma Scan Pro Software (rel. 5.0, Jandel Scientific, Erkrath, Germany). Histograms representing PSDs in terms of volumetric cumulative were best fitted using Microcal Origin Software (rel. 8.5, Microcal Software, Inc., Northampton, MA).

SAA coprecipitates were also characterized by Energy-dispersive X-ray spectroscopy (EDX) microanalysis. Elemental analysis and element mapping were performed using the FESEM equipped with an energy dispersive X-ray spectroscopy (EDX, INCA Energy 350, Oxford Instruments, Witney, United Kingdom).

The loading of NMPs in the polymeric matrix was determined by thermogravimetric analysis (TGA) (SDT Q600, TGA/DSC, TA Instruments, USA), as the residue remaining after the total combustion of the organic component of the particles. The sample was placed in alumina crucibles and heated from 25 up to 600 °C at a rate of 10 °C/min, under a constant air flow of 100 Ncm<sup>3</sup>/min).

Solid state analysis of the samples (XRPD = X-ray powder diffraction) was performed using an X-ray diffractometer (mod. D8 Discover, Bruker AXS, Inc., Madison, WI) with a Cu sealed tube source. Samples were placed in the holder and flattened with a glass slide to assure a good surface texture. The measuring conditions were as follows: Ni-filtered CuK $\alpha$  radiation,  $\lambda = 1.54$  Å,  $2\theta$  angle ranging from 2 to 90° with a scan rate of 3s/step and a step size of 0.02°.

Drug loading was performed dissolving Samples of AMP/CH powder under vigorous stirring in a solution water-acetic acid 2% v/v (pH 2.24) at 37 °C. The solution was stirred for 5 min and stored for 24 h at room temperature to obtain the complete release of the drug from the carrier. The obtained solution was filtered to eliminate the NMPs and CH residue and diluted with water to increase the pH and analysed by HPLC-UV/vis (Hewlett-Packard model G131–132, USA). The column used is a reverse phase C18 column (4.6  $\times$  250 mm; 5 mm particle size; 80Å pore size; Hypersil BDS RP-C18); it was equilibrated at a flow rate of 0.5 mL/min with a mobile phase consisting of phosphate buffer pH 5.0 and acetonitrile (ratio 70:30 v/v). AMP was monitored at 225 nm with a retention time of 5–6 min. Loading efficiency was calculated as the ratio of the drug content of the produced particles over the drug loaded at the beginning.

The drug release rate was analysed by UV/vis spectrophotometer (model Cary 50, Varian, Palo Alto, CA) using a physiologic solution (PS) at pH 6.8 as dissolution medium. 40 mg of powder was charged into a tea bag filter and immersed in 200 mL of PS continuously stirred at 200 rpm and 37 °C. Absorbance at wavelength 264 nm was monitored as: every 1 min for 1 h, every 10 min for 1 h, every

30 min for 8 h, every 60 min for 72 h. Then, the absorbance values were converted into AMP concentration, using a calibration curve.

### 3 Results and Discussion

The system processed by SAA was a suspension of NMPs in an aqueous solution of CH and AMP; therefore, preliminary studies were performed on the suspension in terms of stability of NMPs dispersion and degradation of the drug.

#### 3.1 Stability of Suspensions

For a successful SAA precipitation, it is relevant to have a stable suspension, that does not split before precipitation. For this reason, in a previous work, a study on the suspension stability has been performed [32]. The suspensions were considered useful for SAA processing if they were stable for at least 30 min. Such suspensions were obtained at a chitosan concentration of 10 mg/mL plus 2% Tween 80 (w/w surfactant/solvent) with a maximum loading of 20% of  $\text{Fe}_3\text{O}_4$  nanoparticles (w/w NMPs/chitosan) and at a chitosan concentration of 20 mg/mL plus 7.5% Tween 80 (w/w surfactant/solvent) with a maximum load of 40% NMPs (w/w NMPs/chitosan).

#### 3.2 Micronization Experiments

The micronization of the system AMP/CH by SAA has been studied in a previous work [34] and several operating conditions have been investigated. Based on the previous results [32], the operating conditions for the experiments performed in this work have been chosen as reported in Table 1. For all the experiments a ratio between  $\text{CO}_2$  and liquid solution (gas to liquid ratio = GLR) of 1.8 have been used.

**Table 1** Operating conditions of SAA experiments varying the global concentration, R = AMP/CH, conc = solute concentration,  $P_m$  = mixer pressure,  $T_m$  = mixer temperature,  $P_p$  = precipitator pressure,  $T_p$  = precipitator temperature, Q = flow rate

Conc. CH (mg/mL)	Conc. AMP (mg/mL)	R (wt/wt)	$T_m$ (°C)	$P_m$ (bar)	$T_p$ (°C)	$P_p$ (bar)	$Q_{\text{suspension}}$ (mL/min)	$Q_{\text{CO}_2}$ (gr/min)
10	5	1/2	90	90	113	1.6	4.48	8.06
10	2.5	1/4	91	99	112	1.8	4.62	8.31
10	1.67	1/6	80	109	113	1.1	4.41	7.94

**Fig. 2** Example of powder obtained by SAA of coprecipitate AMP/CH containing 40% di  $\text{Fe}_3\text{O}_4$  (wt/wt<sub>CH</sub>)



The following SAA process parameters have been selected: static mixer temperature and pressure at 90 °C and 100 bar, respectively; precipitator temperature set at 110 °C and a concentration of CH of 10 mg/mL. 40% wt of NMPs have been suspended in the starting solution and AMP/CH ratios of 1/2, 1/4, 1/6 have been studied.

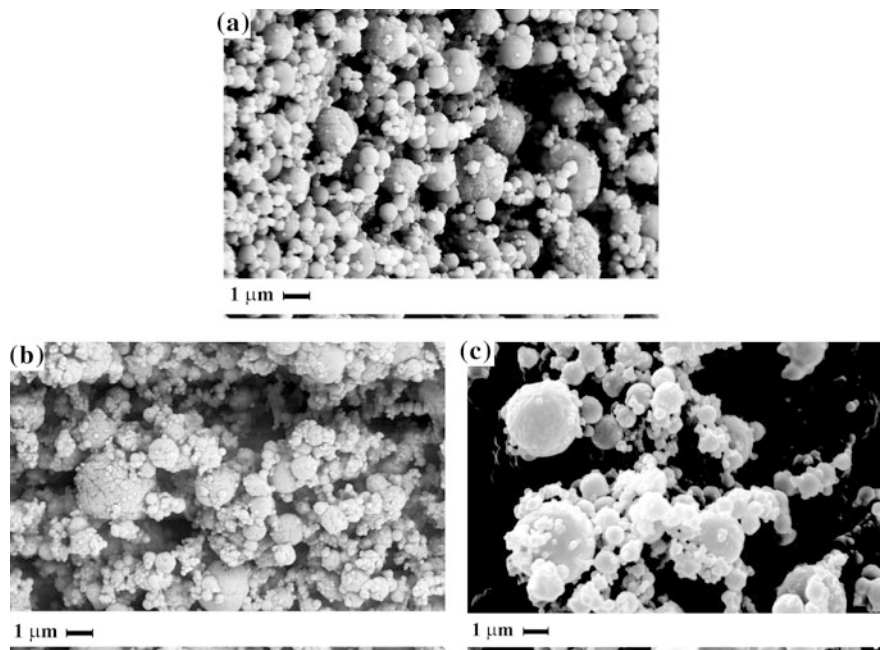
After each experiment, a dry powder with a brownish colour (Fig. 2) was collected on the filter of the precipitator, giving a first evidence of the presence of NMPs uniformly distributed in the particles.

In all the experiments the particles obtained showed a spherical morphology, as in the Fig. 3, that reports, as an example, SEM photomicrographs of coprecipitates at AMP/CH ratios 1/2, 1/4, 1/6 (wt/wt). By increasing CH content a characteristic wrinkling of the particle surface can be noted.

In Fig. 4 particle size distributions in terms of cumulative volumetric percentages are reported, showing that the mean diameter ranges between 0.5 and 1  $\mu\text{m}$  and the maximum observed particle size is about 2  $\mu\text{m}$ . The results did not show a clear influence of drug/polymer ratio on the particle size and distribution in the case of  $R = 1/2$  and  $R = 1/4$ , and there is a slight increase of particle size in case of  $R = 1/6$ .

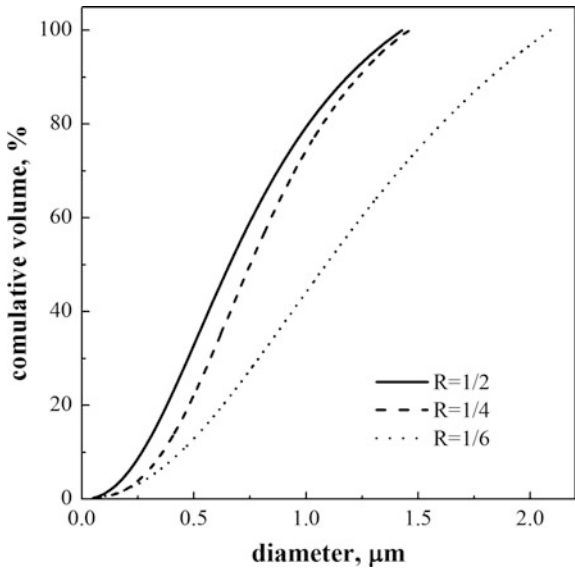
The obtained microspheres have been characterized by several analytical techniques to verify the efficiency of SAA micronization. XRPD analyses of the coprecipitates showed the presence of the characteristic halo typical of an amorphous material and  $\text{Fe}_3\text{O}_4$  crystal peaks (Fig. 5). Therefore, the microspheres are amorphous, the drug is intimately mixed with the polymer and, during the precipitation, the NMPs do not modify their characteristic crystalline structure: they are simply entrapped in the polymeric matrix.

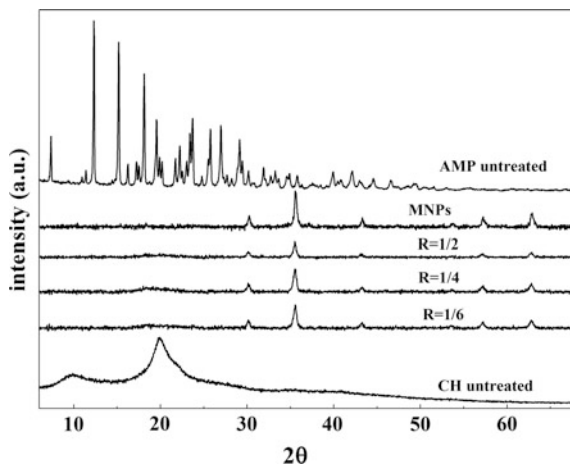
EDX microanalysis allowed to identify the elemental composition of the particles in a SEM photomicrograph, showing the presence of the elements characteristic of the three compounds (Fig. 6). Oxygen (white in Fig. 6) is present in all the compounds, Sulphur (yellow in Fig. 6) is present only in the AMP molecule, as can be observed in Fig. 1 reporting the molecular structure, and Iron (cyan in Fig. 6) is present only in  $\text{Fe}_3\text{O}_4$ . Figure 6 confirms that the three elements are present in the



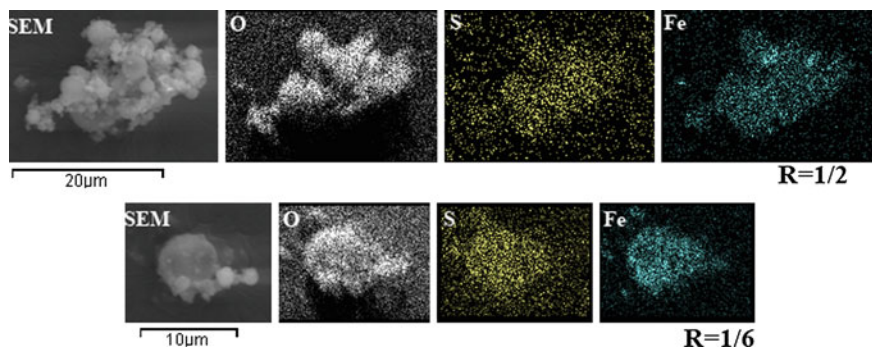
**Fig. 3** SEM photomicrographs of SAA coprecipitate containing 40% di Fe<sub>3</sub>O<sub>4</sub> at different R = AMP/CH: **a** R = 1/2, **b** R = 1/4, **c** R = 1/6

**Fig. 4** PSD of AMP/CH loaded with 40% NMPs micronized by SAA at different R (AMP/CH ratio, wt/wt), in terms of cumulative volumetric percentage. *Dashed line* R = 1/6, *Continuous line* R = 1/4, *Dotted line* R = 1/2





**Fig. 5** XRPD spectra of powders: comparison among untreated materials and microspheres produced by SAA



**Fig. 6** EDX microanalysis of SAA coprecipitated microparticles loaded with 40%  $\text{Fe}_3\text{O}_4$ . Particulars of: SEM image of the analysed areas; Oxygen maps (white); Sulphur maps (yellow); Iron maps (cyan). R = AMPt/CH ratio, wt/wt

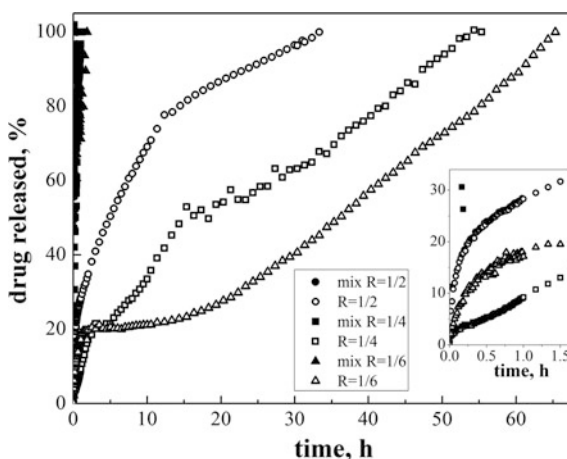
coprecipitates; therefore, AMP and NMPs are loaded in the CH particles; furthermore, the key chemical elements are uniformly distributed in the microparticles, confirming that AMP and CH are intimately mixed in each particle at nanometric level. Furthermore, it is shown that the NMPs are also uniformly distributed in the microparticles, mainly as a result of the stability of the suspension during the micronization process.

To obtain a quantitative information about the NMPs present in the microparticles, TGA analysis has been performed on the coprecipitates obtained by SAA producing the combustion of the organic material. The organic components CH and AMP decompose by combustion, leaving the inorganic residue  $\text{Fe}_3\text{O}_4$ . The quantity



**Table 2** Loading efficiency of AMP and NMPs in SAA coprecipitates.  $\text{Fe}_3\text{O}_4$  starting concentration 40%

Conc. CH (mg/mL)	R = AMP/CH wt/wt	$\text{Fe}_3\text{O}_4$ loading efficiency (%)	$\text{Fe}_3\text{O}_4$ loaded (wt%)	AMP loading efficiency (%)	AMP loaded (wt%)
10	1/2	37	14.81	63	20.95
10	1/4	31	12.47	66	13.24
10	1/6	31	12.25	99	14.12

**Fig. 7** Release profiles from microspheres produced by SAA and physical mixtures at different drug loadings; R = AMP/CH ratio wt/wt, mix = physical mixture

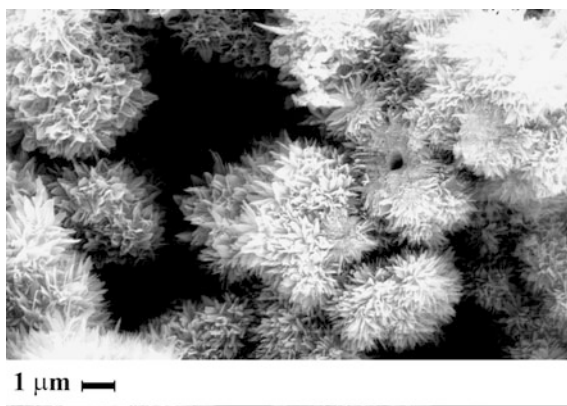
of NMPs loaded in the microparticles varies between 31 and 37%, as reported in Table 2.

HPLC analyses showed that a loading efficiency of the active principle in the range of 63–99%, as reported in Table 2. AMP loading efficiency increases with the decrease of the AMP/CH ratio; this is because the increase of the relative amount of CH improves the entrapment of the active principle.

Drug release studies have been performed using a filter bag in physiologic solution at pH 6.8 and 37 °C. CH is very soluble at very low pH values, on the contrary, at pH close to neutral, characteristic of human tissue, it has a very low solubility and can be used for delayed release. The controlled drug release studies were elaborated considering the measured drug loading: the maximum percentage of drug released from the microspheres was normalized on the drug effectively loaded in the coprecipitate. The release from microspheres was compared with the release from physical mixtures of AMP, CH and NMPs at the same ratios than in the microspheres.

As can be noted in Fig. 7, physical mixtures release the drug within 55 min, mainly because AMP is not entrapped in the CH and dissolves in the aqueous environment. The release of AMP from CH microspheres is slower compared to the physical mixtures, and the release rate is controlled by the AMP/CH ratio. It can be

**Fig. 8** SEM photomicrographs of SAA coprecipitate containing 40% di  $\text{Fe}_3\text{O}_4$  at  $R = 1/4$ , after the AMP release



observed for release of AMP at all the ratios studied a fast release in the first 150 min, followed by a slow release, that is different for the AMP/CH ratios: the higher the amount of CH, the slower the release.

The drug is completely released after about 33 h for  $R = 1/2$ , after about 55 h for  $R = 1/4$  and after about 65 h for  $R = 1/6$ . The observation of the morphology of the microparticles, after the release tests, showed that the particles were dissolved and moved to a gel-like structure.

SEM observation of the microspheres, showed that the presence of NMPs affected the release: the morphology of particles showed that the NMPs opened several holes on their surface during their release (Fig. 8).

## 4 Conclusions

Composite microparticles chitosan-ampicillin loaded with  $\text{Fe}_3\text{O}_4$  nanoparticles were successfully produced by SAA and can be used for the production of pharmaceutical formulation for the targeted release of the active principle in specific sites. The spherical morphology and controlled PSD with a maximum diameter of about  $2\text{ }\mu\text{m}$  allow to use the microspheres for different biomedical applications. High loading efficiency of AMP in CH microspheres can be obtained. The release of the active principle can be controlled by the AMP/CH ratio and there is no interference of NMPs to the release of AMP from the formulation. A starting burst effect followed by a sustained release allow the formulation to be effective up to 72 h for a AMP/CH 1/6.

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