

1 **Microspheres loaded with polysaccharide nanoparticles for pulmonary delivery:**  
2 **Preparation, structure and surface analysis**

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15 **Abbreviations:**

16 A459: adenocarcinoma human alveolar basal epithelial cells

17 Calu-3: human airway epithelial cell line

18 CLSM: confocal laser scanning microscopy

19 CS: chitosan sodium hydrochloride

20 GlcA: glucuronic acid

21 GlcNAc: N-acetyl glucose amine

22 Glc: glucose amine

23 1D-CPMAS <sup>13</sup>C: cross polarization magic angle spinning NMR

24 HA: hyaluronic acid

25 16HBE14o: human bronchial epithelial cell line

26 M: mannitol microspheres

27 M-NPs: microencapsulated nanoparticles

28 Mix: physical mixture of chitosan with hyaluronic acid

29 NMR: nuclear magnetic resonance

30 NPs: nanoparticles  
31 SEM: scanning electron microscope  
32 TEM: transmission electron microscopy  
33 TOF-SIMS: time-of-flight secondary ion mass spectroscopy  
34 TPP: pentasodium tripolyphosphate  
35 XPS: X-ray photoelectron spectroscopy

37

38

### 39 **Abstract**

40 In this work, we report the preparation of a nanoparticle-based dry powder for pulmonary  
41 administration. Hybrid chitosan/hyaluronic acid nanoparticles were produced by ionotropic gelation  
42 and characterized for their physicochemical properties, being further studied by solid nuclear  
43 magnetic resonance (NMR). Using mannitol as carrier, nanoparticles were microencapsulated by  
44 spray drying, resulting in a dry powder with appropriate aerodynamic properties for lung delivery.  
45 In order to investigate the nanoparticles distribution within the carrier matrix, several techniques  
46 were applied that permitted an in-depth analysis of the system structure and surface, such as  
47 confocal laser scanning microscopy (CLSM) and X-ray photoelectron spectroscopy (XPS) in  
48 combination with time-of-flight secondary ion mass spectroscopy (TOF-SIMS). Overall, the studies  
49 conducted revealed that nanoparticles are homogeneously distributed through mannitol  
50 microspheres, suggesting the success of the microencapsulation process. In the light of these  
51 findings, it was concluded that the developed delivery system holds great potential for lung delivery  
52 of macromolecules.

53

54 *Key-words:* Chitosan; Hyaluronic acid; Microspheres; Nanoparticles; Pulmonary administration;  
55 Spray drying; TOF-SIMS; XPS.

56

## 57 **1. Introduction**

58 Presently, there is particular research interest in pulmonary delivery of drugs, specially peptides,  
59 proteins and genes, not only for local, but also for systemic effect. This is primarily due to the  
60 important advantages offered by the pulmonary route, such as the large alveolar surface available  
61 for absorption, very thin diffusion path to the blood stream, extensive vascularisation, relatively low  
62 metabolic activity compared to other routes and avoidance of gastrointestinal degradation and  
63 hepatic metabolism (Agu, Ugwoke, Armand, Kinget, & Verbeke, 2001; Courier, Butz, &  
64 Vandamme, 2002).

65 In spite of this, in order to succeed in the pulmonary delivery of any therapeutic molecules,  
66 many obstacles and lung defense mechanisms, that could hinder the path of foreign substances,  
67 must be overcome, such as the effect of the airways' structure, mucociliary clearance and  
68 phagocytosis by alveolar macrophages (Courier, Butz, & Vandamme, 2002; Hastings, Folkesson,  
69 & Matthay, 2004). Thus, to evade the impact of such barriers and to assure optimal drug delivery to  
70 the desired site, it is critical to develop the appropriate drug carriers. In this respect, specific  
71 characteristics are required which provide the drug delivery system with the ability to reach the  
72 alveolar region, if a systemic effect is desired, or another specific site, or if a local action is intended  
73 (Pandey, & Khuller, 2005). Considering the specific anatomy of the airways, it is traditionally  
74 believed that droplets and/or particles with an aerodynamic diameter within the range of 1-3  $\mu\text{m}$   
75 will present appreciable deposition in the alveolar region, while those with a higher aerodynamic  
76 diameter will mainly deposit in the upper regions (Chrystyn, 1997). Therefore, size and density of  
77 the delivery system are the most critical parameters in obtaining adequate therapeutic effects.  
78 However, notwithstanding the referred aerodynamic requirements, nanoparticles have also been  
79 recently proposed for the same end (Dailey et al., 2003; Sung et al., 2007; Yang et al., 2008, Bailey  
80 et al., 2009) due to their ability to delay or avoid mucociliary clearance and macrophage capture  
81 (Schürch, Gehr, Im Hof, Geiser, & Green, 1990).

82 The selection of suitable biocompatible materials (polymers, lipids, sugars) used for the  
83 preparation of lung carriers has been shown to be an essential consideration and, in this context, the  
84 polysaccharides chitosan and hyaluronan are particularly attractive polymers. Chitosan, a natural  
85 polysaccharide derived from chitin and is one of the most promising materials for transmucosal  
86 drug delivery, given its reported low toxicity, biodegradability and biocompatibility (Hirano, Seino,  
87 Akiyama, & Nonaka, 1988; Issa, Koping-Hoggard, & Artursson, 2005; Varshosaz, 2007), as well as  
88 mucoadhesivity (Lehr, Bouwstra, Schacht, & Junginger, 1992; Agnihotri, Mallikarjuna, &  
89 Aminabhavi, 2004) and enhancement of macromolecules permeation (Bernkop-Schnürch, Kast, &  
90 Guggi, 2003), thus being extensively employed in the development of micro- and nanocarriers  
91 (Grenha et al., 2008). In fact, chitosan is known to be degraded by mammalian enzymes such as  $\alpha$ -  
92 amylase (Muzzarelli, 1997) and lysozyme and has been demonstrated to induce low or absent  
93 toxicity in cell lines representative of the pulmonary route (16HBE14o-, Calu-3 and A549) (Lim,  
94 Forbes, Martin, & Brown, 2001; Florea, Thanou, Junginger, & Borchard, 2005; Grenha, Grainger,  
95 Dailey, Seijo, Martin, Remuñán-López, & Forbes, 2007). Hyaluronic acid is a natural, linear and  
96 non-sulfated glycosaminoglycan (Stern et al., 2007; Bastow et al., 2008; Theocharis et al., 2008;  
97 Volpi et al., 2009), which is present in human tissues and fluids, mostly in soft connective tissue.  
98 Interestingly, it can be found on the surface of alveolar epithelial cells, providing protection against  
99 tissue damage and injury in a number of respiratory diseases (Jiang, Liang, & Noble, 2007) and  
100 preventing pleural thickening in tuberculosis patients (Zhuo, Guo, & Tang, 2003; Cantor, & Turin,  
101 2004).

102 Furthermore, it has been widely implicated in the development of drug and gene delivery  
103 systems directed to different routes of administration ( Lim et al., 2002; Coradini et al., 2004; Peer  
104 et al., 2004; Brown et al., 2005; Woo et al., 2007; Hwang et al., 2008; Gómez-Gaete et al., 2008;  
105 Sahiner et al., 2008; Xin et al., 2010). This interesting profile of hyaluronic acid arises from its  
106 unique characteristics, such as endogenicity, biodegradability, mucoadhesivity (Avitabile et al.,  
107 2001; Morimoto et al., 2001; Mayol et al., 2008; Sivadasa., 2008), the capacity to increase drug

108 circulation time *in vivo* (Peer et al., 2004; Jiang et al., 2008), and the ability to modify drug  
109 dissolution and absorption (Chono, Li, Conwell, & Huang, 2008). Interestingly, hyaluronic acid  
110 selectively binds to CD44 receptors expressed on lung epithelial cells and over-expressed in cancer  
111 cells, a capacity that has prompted its use for targeting purposes (Akima et al., 1996; Taetz et al,  
112 2009). More specifically, it has been employed in drug inhalation and gene therapy, which have  
113 revealed encouraging *in vitro* and *in vivo* outcomes related to improved bioavailability and  
114 transfection (Akima et al., 1996; Taetz et al, 2009; Surendrakumarm et al., 2003; Rouse; 2007;  
115 Hwang et al., 2008). We recently proposed the preparation of chitosan/hyaluronic acid  
116 nanoparticles using a mild gelation technique (De La Fuente, Seijo, & Alonso, 2008a), which  
117 demonstrated great potential for ocular gene delivery (De La Fuente, et al., 2008a; De La Fuente, et  
118 al., 2008b; De La Fuente, et al., 2010). Furthermore, these nanoparticles have shown to have a  
119 potential application in the treatment of asthma, for heparin administration (Oyarzun-Ampuero,  
120 Brea, Loza, Torres, & Alonso, 2009).

121 It is well known that delivering nanoparticles to the lungs is impractical due to their reduced  
122 dimensions and, hence, low inertia (Sung, Pulliam, & Edwards, 2007; Yang, Peters, & Williams III,  
123 2008). To address these limitations, we have recently proposed the microencapsulation of chitosan  
124 nanoparticles within a micron-sized mannitol inert carrier (Grenha, Seijo, & Remuñán-López, 2005)  
125 as an attempt to improve the nanoparticles stability (nanoparticles are administered in solid state  
126 and, therefore, more stable than the liquid counterpart) and aerosolization pattern, by conferring  
127 adequate aerodynamic properties for proper particle deposition and drug delivery in the lungs  
128 (Azarmi, Tao, Chen, Wang, Finlay, Löbenberg, & Roa, 2006; Sham, Zhang, Finlay, Roa, &  
129 Löbenberg, 2004; Freitas, Müller, & 1998).

130 For a better understanding of the relation between surface properties and biological performance,  
131 it is necessary to characterize in detail the surface of the developed carrier, which entails  
132 determining the composition, structure and distribution of all components present on the surface. It  
133 has been reported, specifically for dry powders, that having information on their surface

134 composition affords the possibility of controlling interparticulate interactions and, thus, enhancing  
135 powder dispersion during inhalation (Bosquillon, Rouxhet, Ahimou, Simon, Culot, Pr at, &  
136 Vanbever, 2004; Bunkera, Daviesa, Chena, James, & Roberts, 2006; Chougule, Padhi, Jinturkar, &  
137 Misra, 2007). Thus, the investigation of surface chemistry of dry powders may be beneficial in the  
138 selection of optimal formulation and process parameters to maintain macromolecule integrity and  
139 aerosolization efficiency which definitively result in high *in vivo* outcomes.

140 Herein, we report the preparation of hybrid chitosan/hyaluronic acid nanoparticles and their  
141 characterization by NMR technique. These nanoparticles were microencapsulated in mannitol  
142 microspheres using the spray drying technique, rendering them adequate for pulmonary delivery.  
143 The mannitol microspheres' structure was observed by confocal laser scanning microscopy (CLSM)  
144 in order to investigate the nanoparticles' spatial distribution within mannitol microspheres  
145 following the microencapsulation process. Their surface was further analyzed by the application of  
146 two surface-sensitive analytical techniques, X-ray photoelectron spectroscopy (XPS) and static  
147 time-of-flight secondary ion mass spectrometry (TOF-SIMS), accurately characterizing their  
148 surface chemical composition and determining the presence of nanoparticles.

149

## 150 **2. Materials and methods**

### 151 *2.1. Chemicals*

152 Ultrapure chitosan in the form of hydrochloride salt (CS) (Protasan<sup>®</sup> UP Cl 113, deacetylation  
153 degree 75-90%, viscosity < 20 mPa.s, molecular weight < 150 KDa) was purchased from FMC  
154 Biopolymers [Norway]; hyaluronic acid (HA) (molecular weight ~ 166 KDa) was provided by  
155 Bioiberica [Spain]; fluorescein sodium salt, phosphate buffered saline tablets (PBS) pH 7.4,  
156 pentasodium tripolyphosphate (TPP) and D-mannitol were supplied by Sigma-Aldrich [Spain] and  
157 Bodipy<sup>®</sup> 630/650-X was provided by Molecular Probes [Netherlands]. Ultrapure water [MilliQ  
158 plus, Millipore Ib rica, Spain] was used throughout. All other chemicals were reagent grade.

159

## 160 *2.2. Preparation of chitosan/hyaluronic acid nanoparticles*

161 Chitosan/hyaluronic acid nanoparticles were produced by a slight modification of the ionotropic  
162 gelation technique previously developed by our group (De La Fuente, Seijo, & Alonso, 2008a;  
163 Calvo, Remuñán-López, Vila-Jato, & Alonso, 1997a; Calvo, Remuñán-López, Vila-Jato, & Alonso,  
164 1997b). Electrostatic interactions were involved in the nanoparticles formation, where the positively  
165 charged amino groups of CS interact with both negatively charged HA and TPP. Briefly, solutions  
166 of TPP and HA in ultrapure water were prepared at concentrations of 0.4-2 mg/mL (w/v) and 2-4  
167 mg/mL (w/v), respectively, and then, equal volumes of both solutions were mixed. Thereafter, 1 mL  
168 of this mixture was added to 3 mL of CS solution whose concentration ranged from 1-1.25 mg/mL  
169 (w/v) and the reaction was maintained for 10 minutes under mild magnetic stirring, resulting in  
170 different formulations of nanoparticles as indicated in Table 1. Nanoparticles formed immediately  
171 and were subsequently isolated for further analysis by centrifugation on a 10  $\mu$ L glycerol layer  
172 [18,000 $\times$ g, 30 min, 15 $^{\circ}$ C, Beckmann Avanti 30, Beckmann, USA], afterwards being re-suspended  
173 in 100  $\mu$ L of purified water after discarding the supernatants.

174 For confocal laser scanning microscopy (CLSM) study, CS was labeled with fluorescein  
175 following the method described by De Campos et al (De Campos, Diebold, Carbalho, Sánchez, &  
176 Alonso, 2004). Nanoparticles were also prepared on a large scale, where the final volume of  
177 nanoparticles suspension was scaled to 40 mL. In this case, centrifugation was performed for 40  
178 min at 18,000  $\times$  g and 15  $^{\circ}$ C.

179

## 180 *2.3. Determination of nanoparticles production yield*

181 The nanoparticles production yield was calculated by gravimetry. Fixed volumes of  
182 nanoparticles suspensions were centrifuged (18,000 $\times$ g, 45 min, 15  $^{\circ}$ C), supernatants were discarded  
183 and sediments of nanoparticles were freeze-dried over 48 h (24 hours set at -34  $^{\circ}$ C and gradual  
184 ascent until 20  $^{\circ}$ C), using a Labconco Freeze Dryer [Labconco, USA] (n=6).

185 The process yield was calculated as follows:

$$\text{Process yield (\%)} = \frac{\text{Nanoparticles weight}}{\text{Total solids (CS + HA + TPP) weight}} \times 100$$

189

#### 190 *2.4. Physicochemical characterization of nanoparticles*

191 The morphological appearance of nanoparticles was examined by transmission electron  
192 microscopy (TEM) [CM 12 Philips, Eindhoven, Netherlands]. The samples were previously stained  
193 with 2% phosphotungstic acid and placed on copper grids with Formvar<sup>®</sup> films for viewing.

194 Measurements of nanoparticles size and zeta potential were performed on freshly prepared  
195 samples, by photon correlation spectroscopy and laser Doppler anemometry, respectively, using a  
196 Zetasizer<sup>®</sup> Nano-ZS [Malvern instruments, Malvern, UK]. For particle size analysis, each sample of  
197 isolated nanoparticles was diluted to the appropriate concentration with ultrapure water. Each  
198 analysis was performed at 25°C at a detection angle of 173°C. For the determination of zeta  
199 potential of the electrophoretic mobility, isolated nanoparticles samples were diluted with 0.1 mM  
200 KCl and placed in an electrophoretic cell, where a potential of ± 150 mV was established. Size and  
201 zeta potential of each formulation were analyzed in triplicate (n=3).

202

#### 203 *2.5. Solid NMR spectroscopy of nanoparticles*

204 Solid-state <sup>13</sup>C CP-MAS NMR spectroscopy experiments were performed at 298 K in an 11.7 T  
205 Varian Inova-750 spectrometer (operating at 750 MHz proton frequency) equipped with a T3  
206 Varian solid probe [Varian, Inc, USA]. Solid NMR samples were prepared in 3.2 mm rotors with an  
207 effective sample capacity of 22 μL which corresponds to approximately 30 mg of the powder  
208 sample. The spectra were processed and analyzed with MestreNova software (Mestrelab Research  
209 Inc.). Carbon chemical shifts were assigned to the carbon methylene signal of solid adamantane at  
210 28.92 ppm.



211 Four samples were analyzed: pure chitosan (CS), pure hyaluronic acid (HA), a physical mixture  
212 of equal weights of CS and HA (Mix.) and chitosan/hyaluronic acid freeze-dried nanoparticles  
213 (NPs) (CS/HA/TPP = 3.75/1/1). For each sample, a 1D-CPMAS  $^{13}\text{C}$  (cross polarization magic angle  
214 spinning) spectrum was acquired under semi-quantitative experimental conditions. The inter-scan  
215 delay was set to 3 s, the number of scans was 8000 and the MAS rate was 15 kHz. Heteronuclear  
216 decoupling during acquisition of the FID was performed with Spinal-64 with the proton field  
217 strength of 70 kHz. The cross polarization time was set to 3 ms. During cross polarization, the field  
218 strength of the proton pulse was set constant to 75 kHz and that of the  $^{13}\text{C}$  pulse was linearly  
219 ramped with a 20 kHz ramp near the matching sideband Prior to the acquisition of the 1D-CPMAS  
220 spectra of the samples, the adamantane sample was used to calibrate the maximum  $^1\text{H}$ - $^{13}\text{C}$  cross-  
221 polarization under the experimental conditions.

222

## 223 *2.6. Preparation of dry powders containing chitosan/hyaluronic acid nanoparticles*

224 Sediments of chitosan/hyaluronic acid nanoparticles (CS/HA/TPP = 3.75/1/1), obtained  
225 following centrifugation of the fresh nanoparticles suspensions, were resuspended in a mannitol  
226 aqueous solution and the resultant suspension of nanoparticles in mannitol was spray dried.  
227 Mannitol solutions were prepared with such concentrations that allowed final  
228 mannitol/nanoparticles to be obtained at ratios of 90/10, 80/20, 70/30 (w/w) and suspensions with a  
229 solid content of 3%. Dry powders were obtained in a one step process by spray drying either  
230 aqueous solutions of mannitol or suspensions of nanoparticles in mannitol using a laboratory scale  
231 drier [Büchi<sup>®</sup> Mini Spray Dryer, B-290, Switzerland]. The spray drying operating conditions were:  
232 two fluids external mixing 0.7 mm nozzle, feed rate of 2.5 mL/min and inlet temperature of  $170 \pm 2$   
233  $^{\circ}\text{C}$ , resulting in outlet temperature of  $111 \pm 2$   $^{\circ}\text{C}$ . The air flow rate and the aspirator rate were  
234 constant at 400 NI/h and 70%, respectively. The resultant spray dried powders were collected and  
235 stored in a dessicator at room temperature until use. Preparation of microspheres for CLSM study  
236 was performed with mannitol labeled with the fluorophore Bodipy<sup>®</sup> 630/650-X<sub>7</sub>, which was then

237 mixed with the fluorescently-labeled nanoparticles (described in section 2.2) and, then co-spray  
 238 dried. The labeling of mannitol with Bodipy<sup>®</sup> was obtained by adding a solution of the fluorophore  
 239 in dimethyl sulfoxide to a mannitol solution (0.32 µg Bodipy<sup>®</sup>/mg mannitol), which was then kept  
 240 under magnetic stirring for 1 hour.

241

## 242 *2.7. Determination of spray drying process yield*

243 Process yield of spray drying process was determined by gravimetry establishing a comparison  
 244 between the weight of resultant dry powder (microspheres) and that of the solids involved in the  
 245 formulation, as follows (n=3):

$$\begin{array}{c}
 \text{246} \qquad \qquad \qquad \text{Microspheres weight} \\
 \text{247} \qquad \text{Process Yield (\%)} = \text{-----} \times 100 \\
 \text{248} \qquad \qquad \qquad \text{Total solids (NPs + Mannitol) weight}
 \end{array}$$

249

250

## 251 *2.8. Microspheres morphological and aerodynamic characterizations*

252 Morphology of microspheres was viewed using a scanning electron microscope (SEM, Leo,  
 253 435VP, UK). Dry powders were placed onto metal plates and a 200 nm thick gold palladium film  
 254 was sputter-coated on to the samples [High resolution Sputter Coater SC7640, Termo VG  
 255 Scientific, UK] before viewing.

256 Aerodynamic diameter measurement was obtained using a TSI Aerosizer<sup>®</sup> LD, equipped with an  
 257 Aerodisperser<sup>®</sup> [Amherst process Instrument, Inc, Amherst, Ma, USA], whose measuring principle  
 258 is based on the measurement of particles time of flight in an air stream, according to the following  
 259 equation (n=3):

$$\begin{array}{c}
 \text{260} \qquad \qquad \qquad \pi d^2 \qquad (V_a - V_p) \qquad \qquad \qquad dV_p \\
 \text{261} \qquad \qquad \qquad C_d \text{ --- } \rho_a \text{ ---} = 1/6 \pi d^3 \rho_p \text{ ---}
 \end{array}$$

263 where  $C_d$ : drag coefficient,  $d$ : particle diameter,  $\rho_a$ : density of air,  $V_a$ : velocity of air,  $V_p$ : velocity of  
264 particle, and  $\rho_p$ : density of particle.

265 Real density was measured using a Helium Pycnometer [Micropycnometer, Quanta Chrome,  
266 Model MPY, 2, USA]. Measurements were performed in triplicate ( $n = 3$ ).

267

## 268 *2.9. Structural characterization of nanoparticle-loaded microspheres using CLSM*

269 Confocal laser scanning microscopy (CLSM) study was conducted to characterize the internal  
270 structure of nanoparticles-loaded microspheres (CS/HA/TPP = 3.75/1/1, NPs/Mannitol = 30/70  
271 (w/w)), using a TCS-SP2 vertical microscope [Leica GmbH, Germany], which collects images  
272 using different fluorescent detectors and using, in this case, two laser lines: argon at 488 nm and  
273 helium-neon at 633 nm. Small samples of the dry powder composed of nanoparticles-loaded  
274 microspheres (fluorescein-labeled nanoparticles and Bodipy<sup>®</sup>-labeled mannitol) were placed on a  
275 glass slide and a drop of immersion oil was added to avoid particle displacement during viewing.  
276 Laser excitation wavelengths of 488 and 633 nm were used to scan the powder, and fluorescent  
277 emissions from fluorescein (emission  $\lambda = 492$ -550 nm) and Bodipy<sup>®</sup> (emission  $\lambda = 650$ -725 nm)  
278 were collected using separate channels. Images were acquired with a magnification of 63x, using an  
279 oil immersion lens (HCX PL APO Ibd. BL 63x/1.40). The gray scale images obtained from each  
280 scan were pseudo-colored green (fluorescein) and red (Bodipy<sup>®</sup>), and overlapped afterward (LCS  
281 Lite, Leica Confocal Software, Leica GmbH, Germany) to obtain a multicoloured image.

282

## 283 *2.10. Microspheres surface analysis using XPS and TOF-SIMS*

284 The surface of microencapsulated nanoparticles (CS/HA/TPP = 3.75/1/1, NPs/Mannitol =30/70  
285 (w/w)), mannitol microspheres and chitosan/hyaluronic acid nanoparticles (CS/HA/TPP = 3.75/1/1)  
286 was analyzed by X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass  
287 spectrometry (TOF-SIMS). To do so, powder samples (microencapsulated nanoparticles and

288 mannitol microspheres) or a small drop of nanoparticles suspension were directly placed on a clean  
289 polished monocrystalline silicon wafer, used as the sample holder. In the latter case, the droplet of  
290 chitosan/hyaluronic acid nanoparticles was allowed to dry at room temperature. Mannitol  
291 microspheres and chitosan/hyaluronic acid nanoparticles were used separately as controls.

292 XPS analysis of the samples was performed using a Thermo Scientific K-Alpha ESCA  
293 instrument [VG Escalab 250 iXL ESCA, VG Scientific, U. K], equipped with aluminum Ka1, 2  
294 monochromatized radiation at 1486.6 eV X-ray source. Due to the non conductor nature of samples,  
295 it was necessary to use an electron flood gun to minimize surface charging. Neutralization of the  
296 surface charge was performed using both a low energy flood gun (electrons in the range 0 to 14 eV)  
297 and a low energy Argon ions gun. The XPS measurements were carried out using monochromatic  
298 Al-K radiation ( $\lambda\nu=1486.6$  eV). Photoelectrons were collected from a takeoff angle of  $90^\circ$  relative  
299 to the sample surface. The measurement was carried out in a Constant Analyzer Energy mode  
300 (CAE) with a 100 eV pass energy for survey spectra and 20eV pass energy for high resolution  
301 spectra. Charge referencing was achieved by setting the lower binding energy C1s photopeak at  
302 285.0 eV C1s hydrocarbon peak. Surface elemental composition was determined using the standard  
303 Scofield photoemission cross sections.

304 The static time-of-flight secondary ion mass spectrometry (TOF-SIMS) analysis was performed  
305 where the mass spectra of the samples were recorded on a TOF-SIMS instrument (TOF-SIMS IV,  
306 Ion-Tof GmbH Germany). Samples were bombarded with a pulsed Bismuth ion beam. The  
307 secondary ions generated were extracted with a 10 KV voltage and their time of flight from the  
308 sample to the detector was measured in a reflectron mass spectrometer. Typical analysis conditions  
309 for this work were: 25 keV pulsed  $\text{Bi}_3^+$  beam at  $45^\circ$  incidence, rastered over  $500 \times 500 \text{ um}^2$ .  
310 Electron flood gun charge compensation was necessary during measurements.

311

### 312 **3. Results and discussion**

#### 313 *3.1. Preparation and characterization of chitosan/hyaluronic acid nanoparticle*

314 As described in the methodology section, chitosan/hyaluronic acid nanoparticles were prepared  
315 by the ionotropic gelation of the positively charged CS, mediated by the interaction with oppositely  
316 charged HA and TPP. As evidenced by TEM microphotographs displayed in Figure 1, nanoparticles  
317 showed a spherical morphology. Table 1 depicts the physicochemical properties of the resultant  
318 nanoparticles, which exhibited a positive zeta potential (+19 - +37 mV) and small sizes in the range  
319 of 173-297 nm. The formulation CS/HA/TPP = 3.75/1/1 (w/w) was selected to conduct all  
320 subsequent experiments as it displayed the highest production yield.

321 NMR is a well established technique for structural and dynamic characterization of molecules  
322 and for the study of organic reactions and processes either in solution, semi-solid or solid states.  
323 The NMR study of relatively high-molecular weight polymers, containing a number of carbon  
324 atoms, usually benefits from the use of solid NMR techniques (Mi, Sung, & Shyu, 2000). In this  
325 regard, the 1D-CPMAS <sup>13</sup>C spectra of these polymers may reveal detailed information relative to  
326 their composition with semi-quantitative results (Metz, Ziliox, & Smith, 1996). ID solid-state NMR  
327 experiments were employed here to verify the cross-linking reaction between CS and HA (Figure 2)  
328 that contributes to nanoparticles formation. More specifically, the technique can be sensitive enough  
329 to subtle changes in the electronic environments of the carbon atoms of CS and HA when they are  
330 ionically cross-linked to generate the nanoparticles. To perform this study, four samples were  
331 analyzed: nanoparticles (CS/HA/TPP = 3.75/1/1 (w/w)), pure CS and HA polymers and the physical  
332 mixture of CS and HA (Mix). The corresponding 1D-CPMAS <sup>13</sup>C spectra are displayed in Figure 3,  
333 each of which contains three broad signals that overlap in the band between 43-110 ppm, which is  
334 the typical region of the sugar ring carbons from C1 to C6. The signals in this band can be assigned  
335 as follows: i) region 95-110 ppm corresponds to the C1 anomeric carbons of the polymer, ii) region  
336 70-95 ppm corresponds to the carbons C2 to C5 of the polymer and iii) region 43-70 ppm  
337 corresponds to the C6 methylene carbons of the polymer. The four spectra also show the  
338 characteristic peak at ca. 174 ppm corresponding to the carboxylate and/or carbonyl acetamide  
339 carbons, as well as the carbon peak at ca. 24 ppm corresponding to the methyl group of the

340 acetamide group. According to these observations, all spectra elucidate the essential pattern of  
341 signals related to polysaccharide structure, but are different in the relative intensities, as depicted in  
342 Table 2. Interestingly, the integration of the signals, in Table 2, is consistent with the structures of  
343 these polymers.

344 According to Figure 3, there is a remarkable difference between the spectrum of nanoparticles  
345 and the other spectra, as signals in the first are considerably broader and extend over a larger region  
346 than the corresponding signals in the other spectra. Moreover, some new signals appear in the  
347 spectrum of nanoparticles, which are indicated with asterisk. The new carbon signals, resonating at  
348 ca. 135 and 205 ppm, correspond to spinning sidebands from the CO carbonyl group at ca. 175  
349 ppm. Their presence is indicative of enhanced chemical shift anisotropy of the CO group. There is  
350 also an additional signal at ca. 19 ppm that is presumably due to a methyl group of acetamide.  
351 These changes occurring to the acetamide group of CS could possibly result from re-organization  
352 due to ionic interactions between the randomly cross-linked rings of CS and HA, with effects utterly  
353 leading to a higher heterogeneity and broadening of signals in the NMR spectrum. Similar  
354 observations were described by others for solid NMR spectra of gel based systems (Saiò, Tuzi, &  
355 Naito, 1998). We can conclude, thereby, that the aforementioned changes detected in the 1D-  
356 CPMAS  $^{13}\text{C}$  spectra could confirm the hypothesis of the mechanism of nanoparticles formation,  
357 which involves cross-linking via electrostatic and hydrophobic interactions between the CS and HA  
358 in addition to that contributed by the TPP cross-linker through the gelation process.

359

### 360 *3.2. Microspheres preparation and characterization*

361 Nanoparticles were co-spray dried with mannitol in a one-step spray-drying process with yields  
362 around 65-70 %. As stated in the introduction, the microencapsulation step envisages the  
363 improvement of nanoparticles aerosolization pattern and lung deposition, which are mainly driven  
364 by the aerodynamic parameters of inhaled particles (e.g. size, density) (Vanbever, Mintzes, Wang,  
365 Nice, Chen, Batychy, Langer, & Edwards, 1999; Larhrib, Martin, Prime, & Marriott, 2003; Minne,

366 Boireau, Horta, & Vanbever, 2008). The resultant microencapsulated nanoparticles were viewed by  
367 SEM (Figure 4), evidencing spherical morphology and demonstrating less tendency to aggregate as  
368 the nanoparticles load increased with respect to mannitol. Microspheres exhibited a real density of  
369  $1.45 \text{ g/cm}^3$  and an aerodynamic diameter of  $2.6 \text{ }\mu\text{m}$  (Table 3), which are suitable characteristics to  
370 achieve deep lung deposition (Bosquillion, Lombry, Pr at, & Vanbever, 2001; Mustante, Schroeter,  
371 Rosati, Crowder, Hickey, & Martonen, 2002).

372 The application of sensitive techniques to characterize the structure of dry powders provides  
373 important information that helps to elucidate the behavior of these drug delivery systems in  
374 subsequent studies, both *in vitro* and *in vivo*. CLSM has been used to this end, since it allows us to  
375 acquire high resolution optical sections of x-y scans along the z-axis, which are then reconstructed  
376 into 3-D multicolored views, enabling a complete visualization of the dry powder external and  
377 internal structure, as well as the spatial arrangement of the components (Lamprecht, Sch afer, &  
378 Lehr, 2000). In our work, the acquisition of fluorescent images by CLSM enabled us to precisely  
379 detect the nanoparticles location within the microspheres. This could not be attained by SEM, which  
380 only provides information on the particles surface structure, rather than its internal structure. Figure  
381 5(a-c) displays the images of microsphere encapsulating chitosan/hyaluronic acid nanoparticles. An  
382 outer shell composed of mannitol (red channel) and an even distribution of chitosan/hyaluronic acid  
383 nanoparticles throughout the microsphere matrix can be observed. The presence of a mannitol outer  
384 shell is confirmed by Figure 5(d), which further evidences microspheres spherical shape as  
385 previously observed by SEM. The homogeneous nanoparticles distribution within the mannitol  
386 microspheres without detecting any punctuate green signals of aggregated particles in the  
387 microspheres matrix may lead to the assumption that mannitol is almost entirely located at the  
388 particles' surface. This was also verified by the optical cross-sections of the confocal images (not  
389 shown) which suggest that the microsphere matrix is almost occupied by the fluorescent  
390 nanoparticles. It has been shown that sugar stabilizers, like mannitol, tend to preferentially adsorb at  
391 the air/liquid interface during the drying process (Arakawa, & Timasheff, 19982; Wang, Chua, &

392 Wang, 2004). Therefore, we may hypothesize that non-specific interactions occurred between the  
393 mannitol and the hydrophobic fluorophore (Bodiby<sup>®</sup>), displacing the positively charged  
394 nanoparticles inwards. It is noteworthy that these findings are similar to those reported in previous  
395 studies for microparticles designed for inhalation therapy (Ely, & Finlay, 2007).

396 XPS and TOF-SIMS represent a complementary approach as non destructive and surface-  
397 sensitive analytic techniques. However, the particular interest of their application in the study of  
398 drug delivery systems arises from the capability of these techniques to provide quantitative and  
399 qualitative information of surface composition (De Vries, E, 1998), which provides valuable  
400 information for the interpretation of kinetic and dynamic behavior, such as drug dissolution,  
401 stability, distribution and release (Chesko, Kazzaz, Ugozzoli, Singh, O'hagan, Madden, Perkins, &  
402 Patel, 2008; Dahlberg, Millqvist-Fureby, & Schuleit, 2008). Additionally, using these tools, the  
403 encapsulation efficiency of microencapsulated drugs (Xie, Marijnissen, & Wang, 2006; Morales,  
404 Ruiz, Oliva, Oliva, & Gallardo, 2007) or nanoparticles (Grenha, Seijo, Serra, & Remuñán- López,  
405 2007).can also be assessed. This latter approach was our goal in the present study. Considering the  
406 fact that microspheres have a lot of surface contact due to their powdery nature, the adsorption of  
407 atmospheric natural contaminants such as nitrogen (N), is highly probable, therefore sample  
408 surfaces were sputter cleaned using a soft argon ion beam (Ar+/1KV, /60 sec, 2X1 mm<sup>2</sup>). The  
409 signals of the contaminating N in the powder samples almost disappeared (preliminary data not  
410 shown), indicating that it was weakly bound (adsorbed) and, thus, easily removed.

411 As displayed in Table 4, however very weak N signals were detected in these samples (values  
412 below 0.1 AT%), which could be explained on the basis that the ionic barrel is 45 degrees to the  
413 surface, generating areas of shadow where the argon ions cannot reach. By contrast, the N signal in  
414 the chitosan/hyaluronic acid nanoparticles spectrum persisted after the sputter cycle with a  
415 relatively high value, suggesting that it is chemically bonded and which could be ascribed to CS.  
416 Moreover and as expected, both Na and P, ascribed to TPP in the nanoparticles, were detected  
417 solely on the surface of chitosan/hyaluronic acid nanoparticles; whereas were absent on the surface



418 of either mannitol microspheres or microencapsulated nanoparticles. Taking into account the  
419 detection limit of XPS (all elements except H:  $\sim 0.01$  monolayer, or  $\sim 0.1\%$  bulk), this finding  
420 indicates the absence of TPP on the powder surfaces. Our assumption of efficient nanoparticles  
421 microencapsulation can be further reinforced by the C/O ratio, which is similar for mannitol  
422 microspheres and the microencapsulated nanoparticles (1.15 and 1.12, respectively). Interestingly,  
423 this ratio is different from that of chitosan/hyaluronic acid nanoparticles (1.6), suggesting that the  
424 surfaces of both mannitol microspheres and the microencapsulated nanoparticles are similar in  
425 terms of the atomic composition and concentrations of C and O. Additionally, the higher C/O ratio  
426 for the chitosan/hyaluronic acid nanoparticles implies lower surface concentration of O, which is  
427 due to the contribution of other elements detected on the surface. It is worth while to notice here  
428 that a signal for silicon was identified in some spectra which could be originated from the silicon  
429 wafer used as a sample support during the analysis (Grenha, Seijo, Serra, & Remuñán- López,  
430 2007).

431 This result was also confirmed by deconvolution analysis of the spectra where the high  
432 resolution spectra of carbon (C1s) signals, showing an envelope, were curve fitted using the  
433 Gaussian distribution into a series of peaks corresponding to different functional groups. We have  
434 assigned as reference the peak at the lowest binding energy (285.0 eV) to carbon atoms linked to  
435 carbon and hydrogen atoms. Table 5 summarizes the relative peak area of each carbon environment.  
436 As can be seen, the peak areas (%) and relative intensities of (C-C, 285 eV), (C-O, 286.8 eV) and  
437 (C=O, 288.37 eV) are nearly similar in mannitol microspheres and microencapsulated  
438 nanoparticles. More importantly, the peak of (O-C=O, 289.54 eV), unique for CS, was detected in  
439 the spectrum of chitosan/hyaluronic acid nanoparticles but not in the other two samples. The  
440 analysis of this result was also confirmed by deconvolution analysis of the spectra where the high  
441 resolution spectra of carbon (C1s) signals, showing an envelope, were curve fitted using the  
442 Gaussian distribution into a series of peaks corresponding to different functional groups. We have

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444 carbon and hydrogen atoms.

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447 nearly similar in mannitol microspheres and microencapsulated nanoparticles. More importantly,  
448 the peak of (O-C=O, 289.54 eV), unique for CS, was detected in the spectrum of  
449 chitosan/hyaluronic acid nanoparticles but not in the other two samples. The analysis of  
450 deconvoluted C1s high resolution spectra re-affirms that chitosan/hyaluronic acid nanoparticles are  
451 entirely encapsulated in mannitol microspheres.

452 TOF-SIMS analysis was conducted under non-destructive energetic conditions and under the  
453 static limit ( $10^{12}$  ions/cm<sup>3</sup>). In the mass spectra, positive ions were detected. According to the  
454 spectra displayed in Figure 6, the nanoparticles spectrum differs from those of both microsphere  
455 samples in the existence of some molecular fragments [ $m/z = 232(\text{C}_{16}\text{H}_{24}\text{O})$ ,  $205(\text{C}_{15}\text{H}_9\text{O})$ ,  
456  $200(\text{C}_{10}\text{H}_{16}\text{O}_4)$ ] in addition to the main and most representative ions of mannitol observed at  $m/z =$   
457 183 (the molecular ion + H) and the two times molecular ion + H observed at  $m/z = 365$ . At the  
458 same time, many identified molecular fragments of nanoparticles, not observed in the spectra of  
459 microspheres and located at  $m/z = 189(\text{C}_7\text{H}_{13}\text{O}_4\text{Si})$ ,  $202(\text{C}_{12}\text{H}_{10}\text{O}_3)$ ,  $215(\text{C}_{11}\text{H}_{12}\text{O}_3\text{Na})$ ,  
460  $239(\text{C}_{15}\text{H}_{11}\text{O}_3)$  and  $226(\text{C}_{18}\text{H}_{10})$ , could result basically from the fragmentation of both CS and HA.  
461 Furthermore, other intensive signals for fragments containing N and O are clearly observed in the  
462 nanoparticles but not in mannitol microspheres and microencapsulated nanoparticles, located at  $m/z$   
463  $= 60(\text{C}_2\text{H}_6\text{NO})$ ,  $59(\text{C}_2\text{H}_5\text{NO})$ ,  $58(\text{C}_2\text{H}_4\text{NO})$  and which could arise from the fragmentation of CS  
464 (Figure 7-1). The N-containing fragment ( $\text{C}_2\text{H}_4\text{NO}$ ,  $m/z = 58$ ), identified as intensive in the  
465 nanoparticles sample, was detected however in both microsphere samples but in one order of  
466 magnitude lower, which is likely due to the atmospheric exposure as mentioned previously (Figure  
467 7-2). Importantly, the  $\text{PO}_3$  fragment ( $m/z = 79$ ), attributed to TPP, is intensive and clearly observed  
468 in the nanoparticles sample whereas it is not detected in that of mannitol microspheres and its

469 intensity in microencapsulated nanoparticles is at least one order of magnitude lower than that of  
470 nanoparticles sample (Figure 8).

471 According to our observations from these spectra, the samples of mannitol microspheres and the  
472 microencapsulated nanoparticles, if not identical, are very similar (intensity of the identified  
473 fragments and also the distribution of the intensity between ions). These outcomes demonstrate that  
474 chitosan/hyaluronic acid are efficiently encapsulated within the mannitol carrier, especially if we  
475 refer to the fact that the TOF-SIMS technique is qualified with the highest surface sensitivity for  
476 surface analysis (detection limit range of ppm-ppb, orders of magnitude better than XPS) and the  
477 resolution depth of 1-3 monolayers.

478

#### 479 **4. Conclusion**

480 Chitosan/hyaluronic acid nanoparticles were prepared, characterized and microencapsulated in  
481 mannitol microspheres, resulting in a dry powder that shows adequate aerodynamic properties for  
482 deep pulmonary deposition. Following the encapsulation process, structural analysis of the dry  
483 powder was provided by CLSM, which elucidated that the nanoparticles were homogeneously  
484 distributed within the mannitol microsphere. The evidence that nanoparticles were completely  
485 encapsulated within the carrier by means of the spray drying process, was achieved by application  
486 of the sensitive surface analysis techniques, XPS and TOF-SIMS. These outcomes confirm the  
487 success of nanoparticles microencapsulation by spray drying. We expect, thereby, that the  
488 microencapsulated nanoparticles hold promise for pulmonary delivery of macromolecules such as  
489 proteins and nucleic acids, as these nanoparticles have demonstrated great potential in gene  
490 transfection in ocular cell lines (De La Fuente, Seijo, & Alonso, 2008b). Therefore, further work is  
491 required to investigate the delivery potential of these developed carriers, in the form of dry powders  
492 in pulmonary cell lines.

493

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501

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Table 1. Process yields and physiochemical properties of chitosan/hyaluronic acid nanoparticles (CS/HA NPs), prepared with different concentrations of hyaluronic acid (HA), tripolyphosphate (TPP) and chitosan (CS) (mean  $\pm$  S.D., n = 3).

CS/HA/TPP (w/w/w)	Yield (%)	Size (nm)	Z potential (mV)
3.75/1/1	56 $\pm$ 8	233 $\pm$ 3	+ 37 $\pm$ 2
4.3/1.1/1	44 $\pm$ 1	239 $\pm$ 4	+ 35 $\pm$ 1
4.3/1.4/1	45 $\pm$ 2	275 $\pm$ 20	+ 34 $\pm$ 1
5.0/1.3/1	41 $\pm$ 4	297 $\pm$ 23	+ 34 $\pm$ 1
5.0/1.7/1	48 $\pm$ 2	212 $\pm$ 4	+ 25 $\pm$ 1
5.0/2.5/1	56 $\pm$ 1	254 $\pm$ 6	+ 23 $\pm$ 1
6.3/3.1/1	40 $\pm$ 4	241 $\pm$ 5	+ 20 $\pm$ 1
7.5/3.8/1	41 $\pm$ 3	219 $\pm$ 7	+ 26 $\pm$ 1
9.4/4.7/1	33 $\pm$ 1	197 $\pm$ 4	+ 25 $\pm$ 1
15.0/10/1	23 $\pm$ 9	173 $\pm$ 1	+ 19 $\pm$ 1

Table 2. Chemical shifts (ppm) and signal integrations obtained from the  $^{13}\text{C}$  1D-CPMAS spectra of the samples studied: pure chitosan (CS), pure hyaluronic acid (HA), physical mixture of both polymers (Mix), and chitosan/hyaluronic acid nanoparticles (NPs) (CS/HA/TPP =3.75/1/1). The relative area of the  $^{13}\text{C}$  NMR signal is indicated between parentheses.

Chemical Shift (ppm)					
Simple	C1 to C6 (sugar) <sup>a</sup>	CO (acetamide) <sup>b</sup>	CO (glucuronic) <sup>b</sup>	CH <sub>3</sub> (acetamide) <sup>c</sup>	CO* <sub>d</sub>
CS	43-110 (27.3)	174.2 (1.0)	---	22.8 (0.8)	---
HA	43-110 (7.26)	173.6 (1.0)		22.6 (0.39)	---
Mix	43-110 (7.75)	173.9 (1.0)		22.6 (0.67)	---
NPs	43-110 (7.75)	174.2 (1.0)		22.6 (0.98)	214.0 (0.31)

a: Integral from 43 to 110 ppm, b: Integral from 163 to 185 ppm, c: Integral from 12 to 34 ppm, d: Integral from 205 to 222

Table 3. Aerodynamic properties of dry powders prepared with different mannitol/ nanoparticles weight ratios and solids contents (CS/HA/TPP = 3.75/1/1, mean  $\pm$  S.D., n = 3).

Mannitol/ nanoparticles ratio	Solids content <sup>a</sup> (%)	Feret's diameter <sup>b</sup> ( $\mu\text{m}$ )	Real density ( $\text{g}/\text{cm}^3$ )	Aerodynamic diameter ( $\mu\text{m}$ )
70/30	3.0	$2.3 \pm 0.7$	$1.45 \pm 0.06$	$2.57 \pm 0.08$
80/20	2.8	$2.7 \pm 1.3$	$1.45 \pm 0.12$	ND
90/10	3.0	$2.2 \pm 0.4$	$1.45 \pm 0.17$	ND

a: Solids content represents the total solids concentration (%) of the spraying suspensions, b: Feret's diameters are determined by optical microscopy

Table 4. Surface elemental composition (atomic %), determined by XPS, of chitosan/hyaluronic acid nanoparticles (CS/HA NPs), mannitol microspheres (M) and microencapsulated nanoparticles (M-NPs) (CS/HA/TPP = 3.75/1/1, mannitol/ nanoparticles = 70/30).

Element (%)	CS/HA NPs	M	M-NPs
C	53.2	52.5	43.0
O	33.5	45.6	38.5
N	5.2	0.10	0.10
Na	1.4	0	0
P	4.6	0	0
Si	1.4	0	12.5
S	0.7	1.7	5.9
ratio C/O	1.6	1.15	1.12

C: carbon, O: oxygen, N: nitrogen, Na: sodium, P: phosphorus. Si: silicon, S: sulpher.



Table 5. The relative peak area (%) of each carbon environment for chitosan/hyaluronic acid nanoparticles (CS/HA NPs), mannitol microspheres (M) and microencapsulated nanoparticles (M-NPs) (CS/HA/TPP = 3.75/1/1, mannitol/nanoparticles = 70/30).

Sample	<u>C-C/C-H</u> 285eV	<u>C-O</u> 286.8eV	<u>C=O</u> 288.4eV	<u>O-C=O</u> 289.5eV
CS/HA NPs	36.0	45.7	14.2	4.1
M	29.3	61.6	9.1	0
M-NPs	39.0	56.1	4.9	0

**Figure legends:**

**Figure 1.** TEM microphotographs of chitosan/hyaluronic acid nanoparticles (CS/HA/TPP = 3.75/1/1).

**Figure 2.** A numbering scheme for hyaluronic acid (above) and chitosan (bottom) polysaccharides where GlcA, GlcNAc, and Glc refer to glucuronic acid, N-acetyl glucose amine and glucose amine, respectively.

**Figure 3.** Solid-state  $^{13}\text{C}$ -NMR spectra of chitosan/hyaluronic acid nanoparticles (NPs) (CS/HA/TPP = 3.75/1/1), chitosan (CS), hyaluronic acid (HA), and the physical mixture of CS with HA (Mix) (CS/HA = 3.75/1). The signal assignment is indicated. In the spectrum of NPs, some signals that are not present in the other samples are labeled with an asterisk and discussed in the text.

**Figure 4.** SEM microphotographs of microencapsulated chitosan/hyaluronic acid nanoparticles (CS/HA/TPP = 3.75/1/1) at different mannitol/nanoparticles theoretical ratios (w/w): (a) 90/10, (b) 80/20, (c) 70/30.

**Figure 5.** Confocal microscopy images of a mannitol microsphere loaded with chitosan/ hyaluronic acid nanoparticles (CS/HA/TPP = 3.75/1/1, mannitol/nanoparticles = 30/70 (w/w): (a) a green channel representing the fluorescently-labeled nanoparticles, (b) a red channel representing mannitol labeled with Bodipy<sup>®</sup>, (c) channels overlapping; and (d) a section of mannitol microspheres containing the nanoparticles with both overlapping channels. The scale bars in panels (a-c) amounts to 4 microns and that in panel (d) amounts to 10 microns.

**Figure 6.** Mass spectra obtained by TOF-SIMS, showing the region of molecular ion + H<sup>+</sup> of mannitol for (a) mannitol microspheres, (b) microencapsulated nanoparticles (CS/HA/TPP = 3.75/1/1, mannitol/ nanoparticles = 70/30 (w/w) and (c) chitosan / hyaluronic acid nanoparticles (CS/HA/TPP = 3.75/1/1).

**Figure 7.** Mass spectra obtained by TOF-SIMS, showing (Fig. 10-1): N and O containing fragments in (a) mannitol microspheres, (b) microencapsulated nanoparticles (CS/HA/TPP = 3.75/1/1, NPs/Mannitol = 30/70 (w/w)), (c) chitosan/hyaluronic acid nanoparticles (CS/HA/TPP = 3.75/1/1); and (Fig. 10-2): the fragment C<sub>2</sub>H<sub>4</sub>NO at m/z = 58 in the same samples.

**Figure 8.** Mass spectra obtained by TOF-SIMS, showing the region of phosphate fragments for the samples: (a) mannitol microspheres, (b) microencapsulated nanoparticles (CS/HA/TPP = 3.75/1/1, mannitol/nanoparticles = 70/30 (w/w), (c) chitosan/hyaluronic acid nanoparticles (CS/HA/TPP = 3.75/1/1).

Figure 1. TEM microphotographs of nanoparticles  
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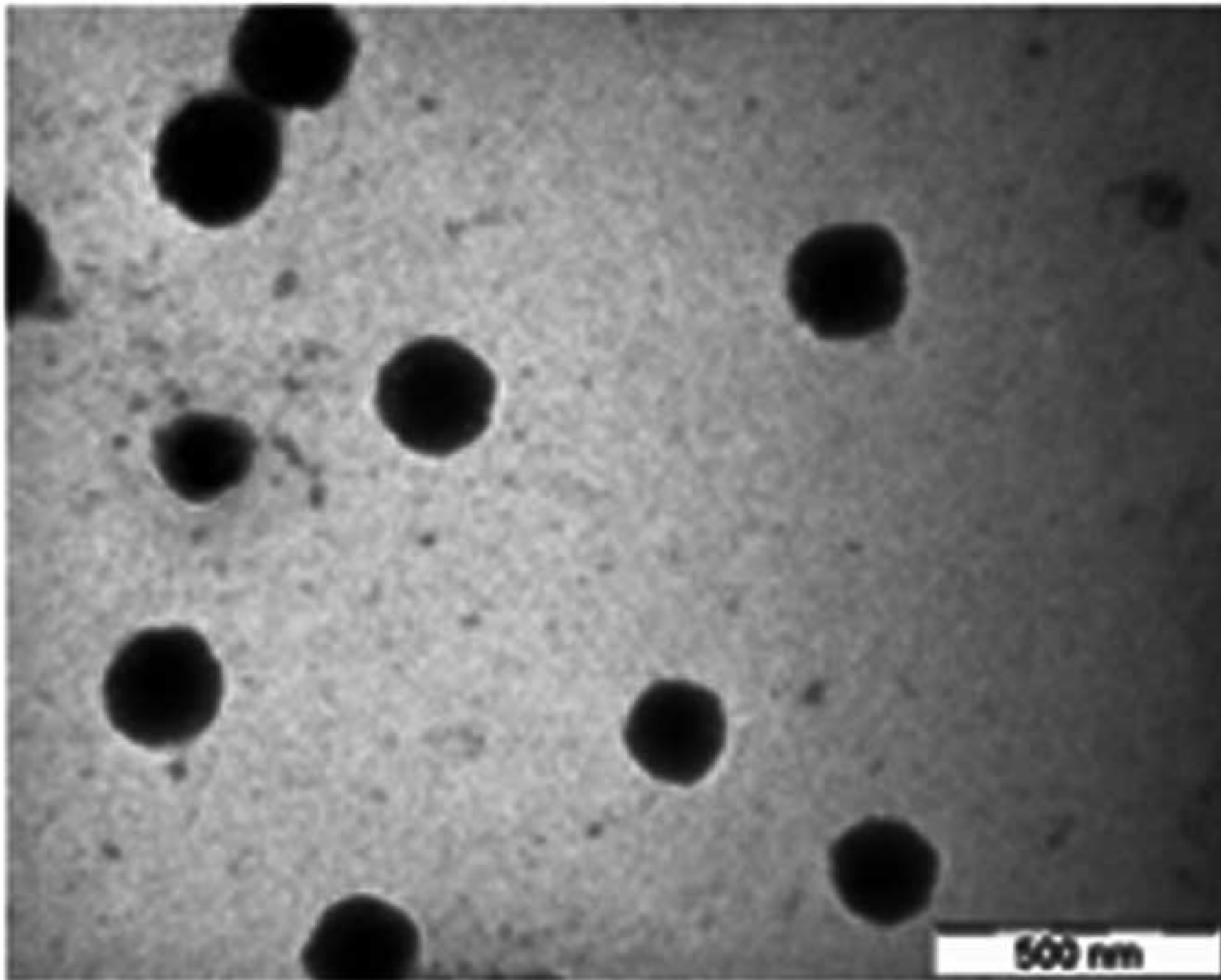


Figure 2. A numbering scheme for hyaluronic acid (above) and ch  
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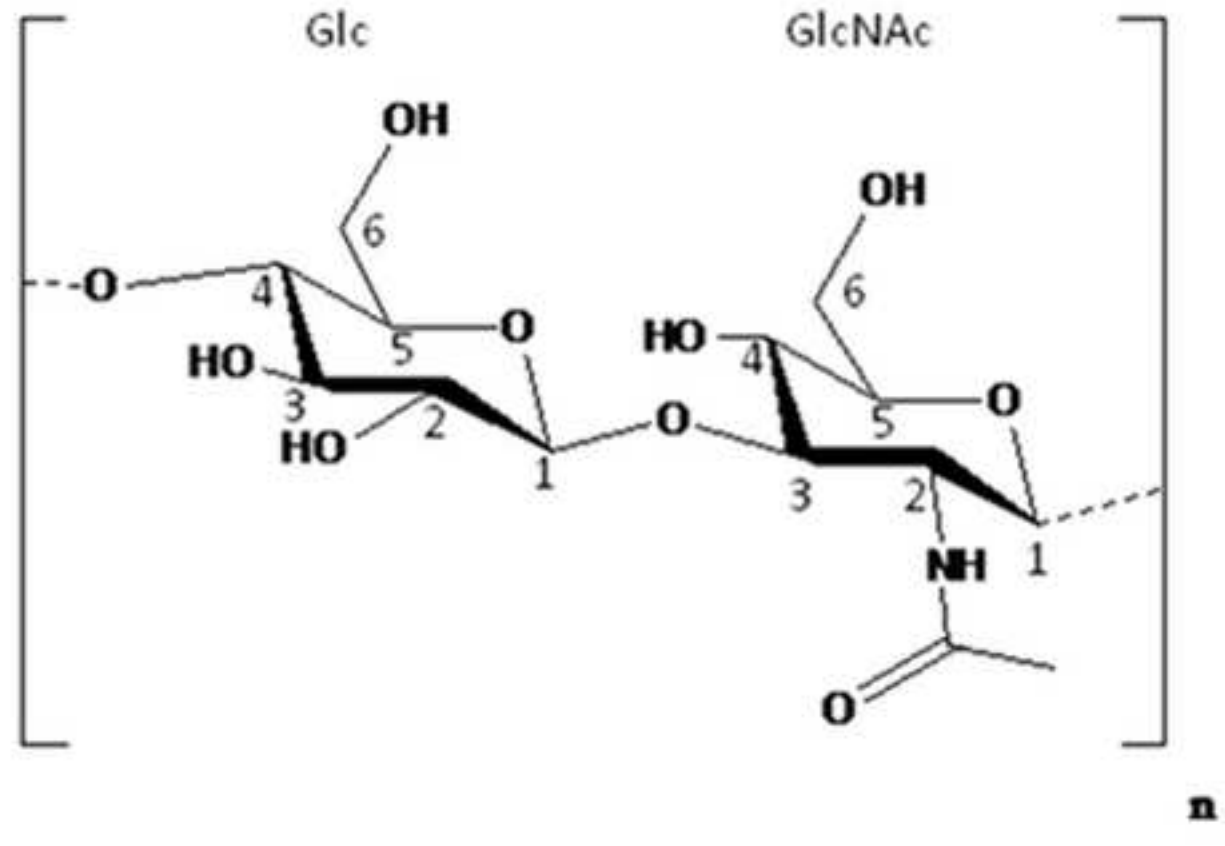
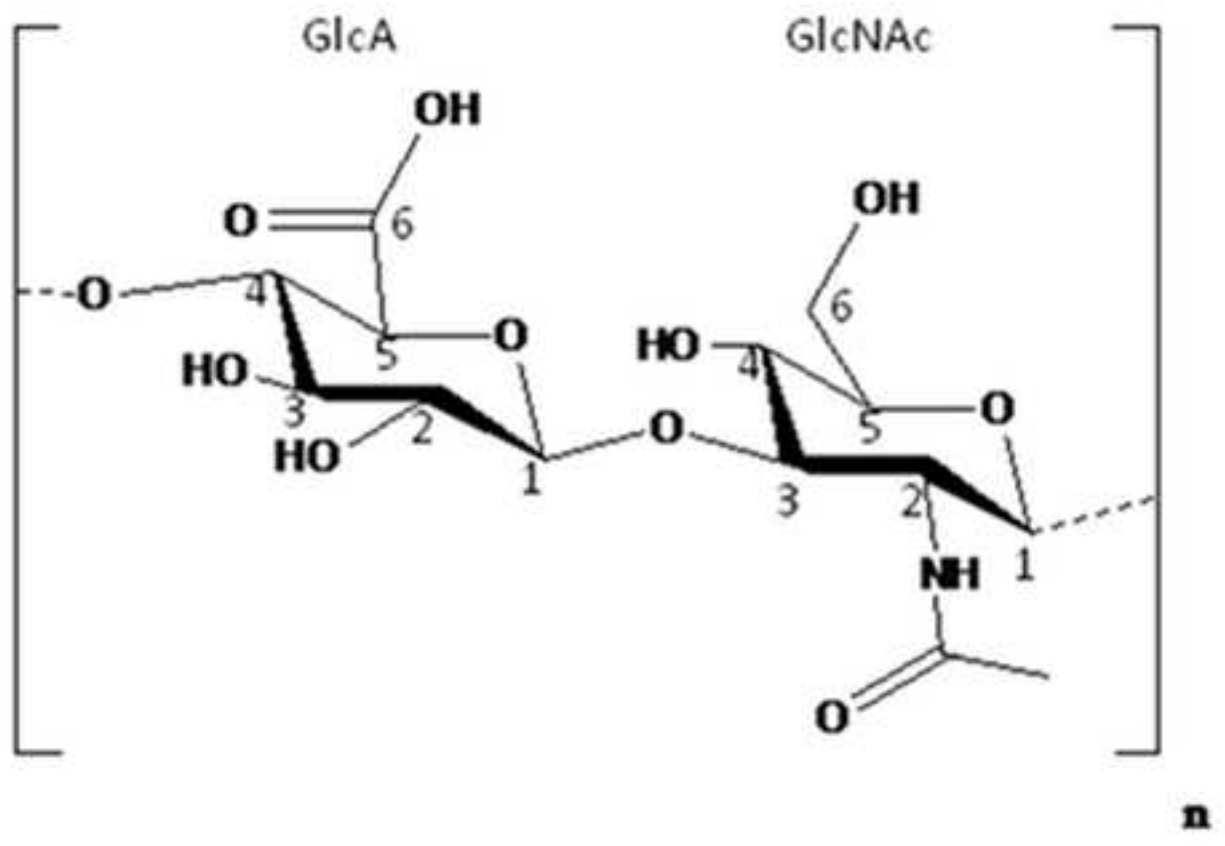


Figure 3. Solid-state  $^{13}\text{C}$ -NMR spectra of chitosan/hyaluronic aci  
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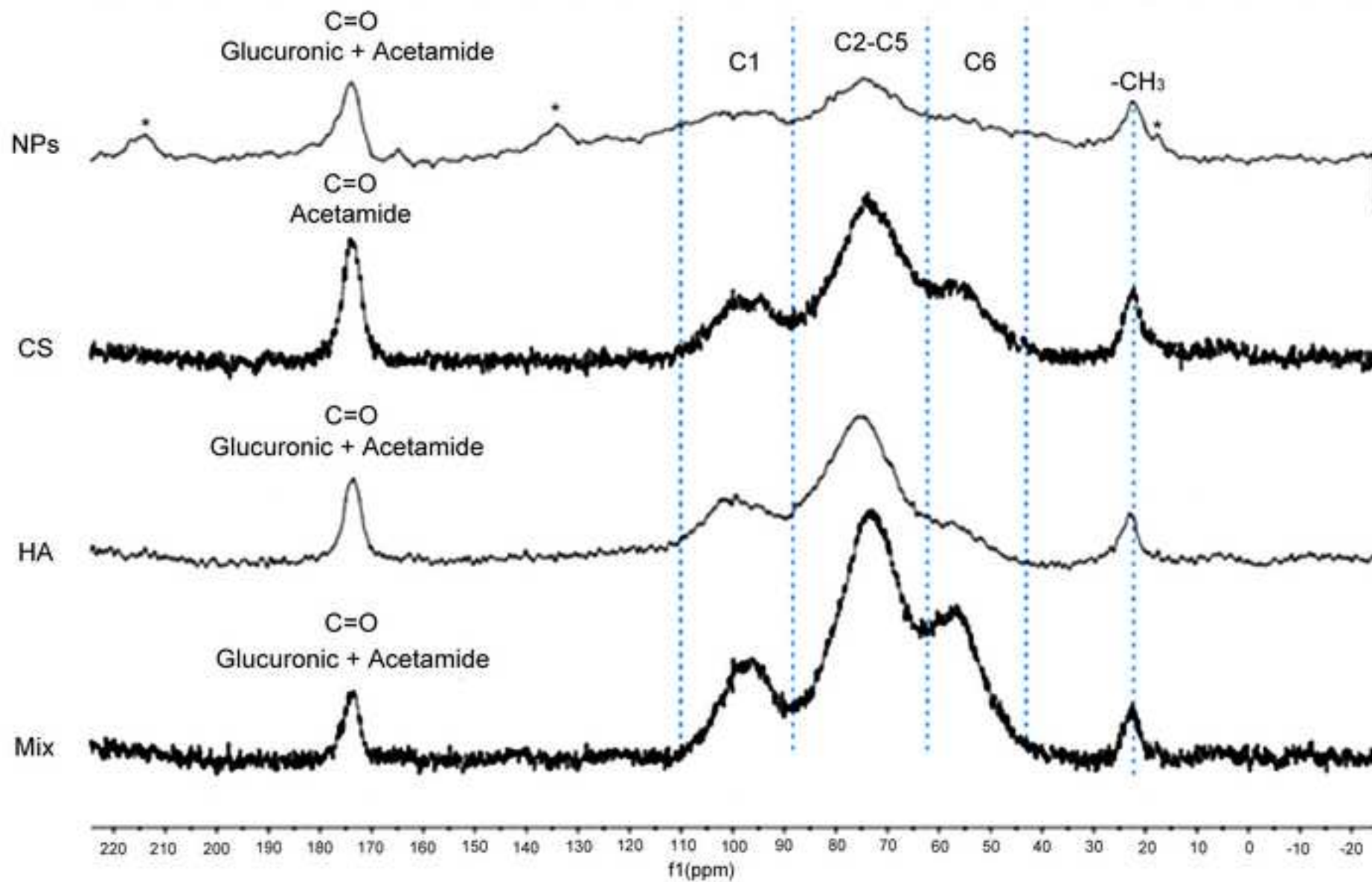


Figure 4. SEM microphotographs of microencapsulated chitosan/hya  
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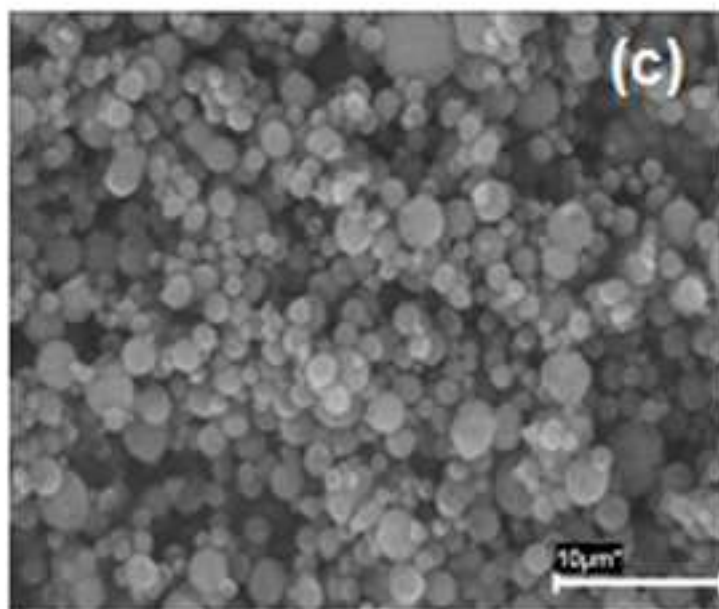
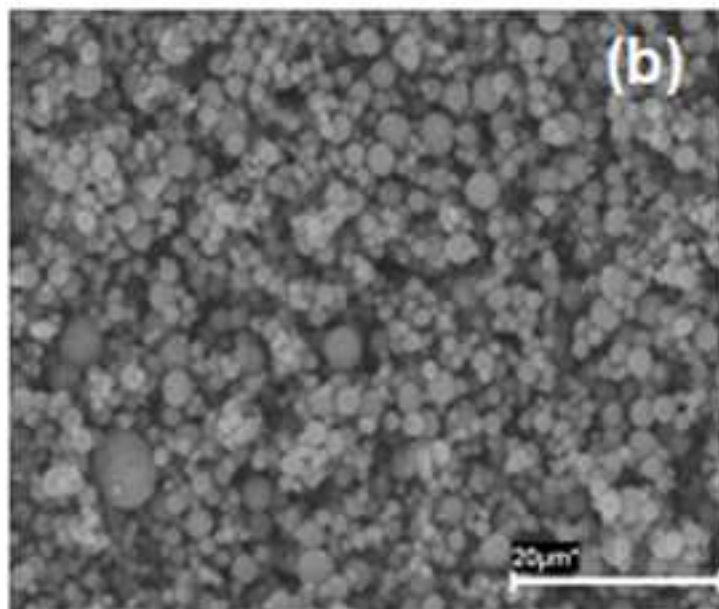
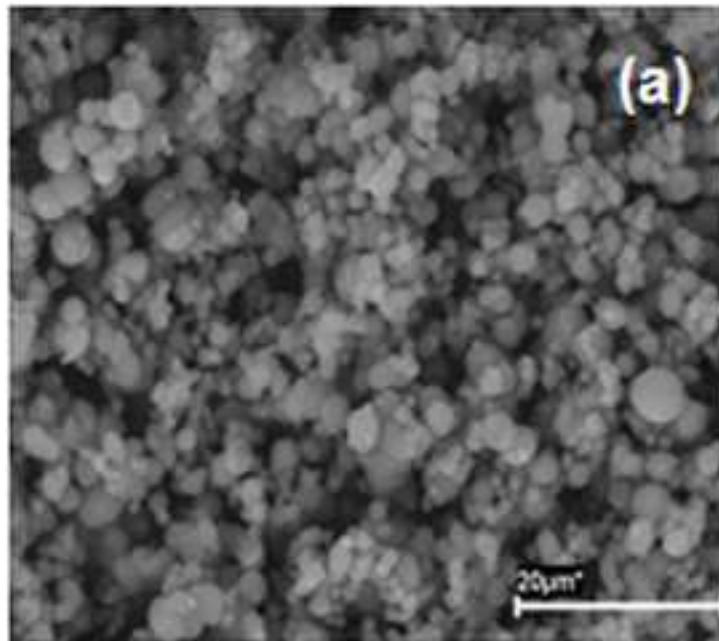


Figure 5. Confocal microscopy images of a mannitol microsphere I  
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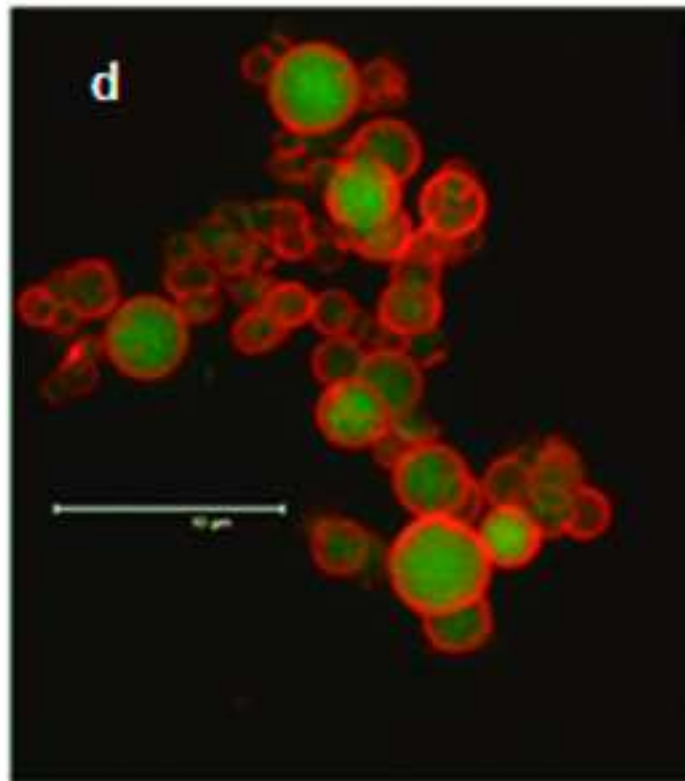
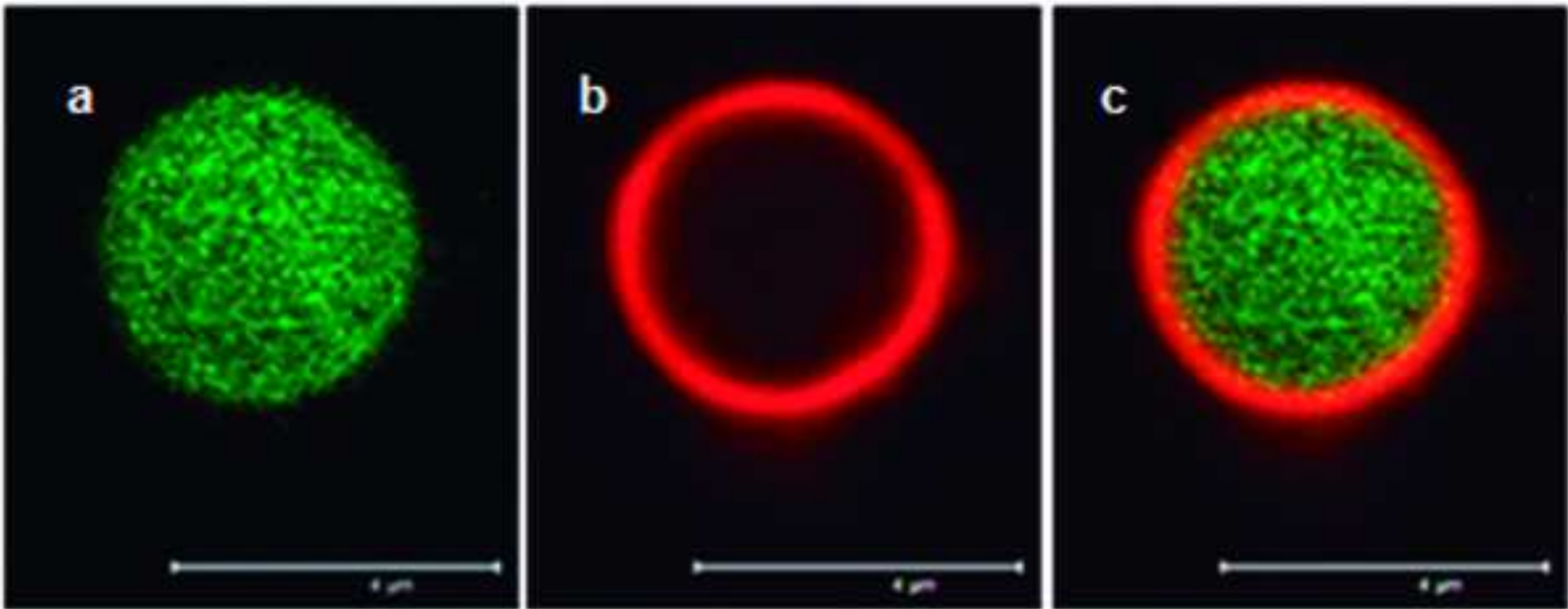




Figure 6. Mass spectra obtained by TOF-SIMS, showing the region  
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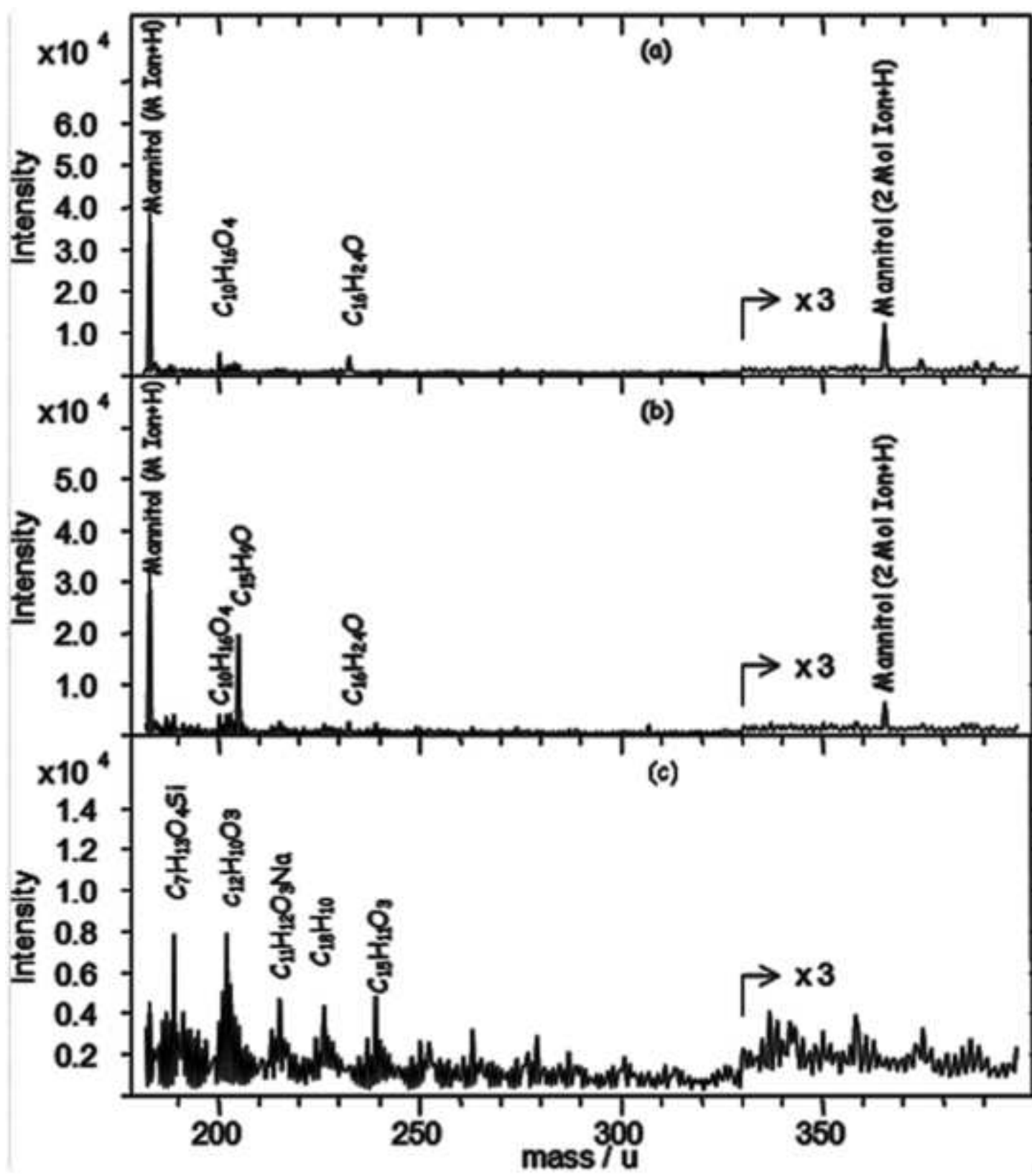


Figure 7. Mass spectra obtained by TOF-SIMS, showing (Fig. 10-1)  
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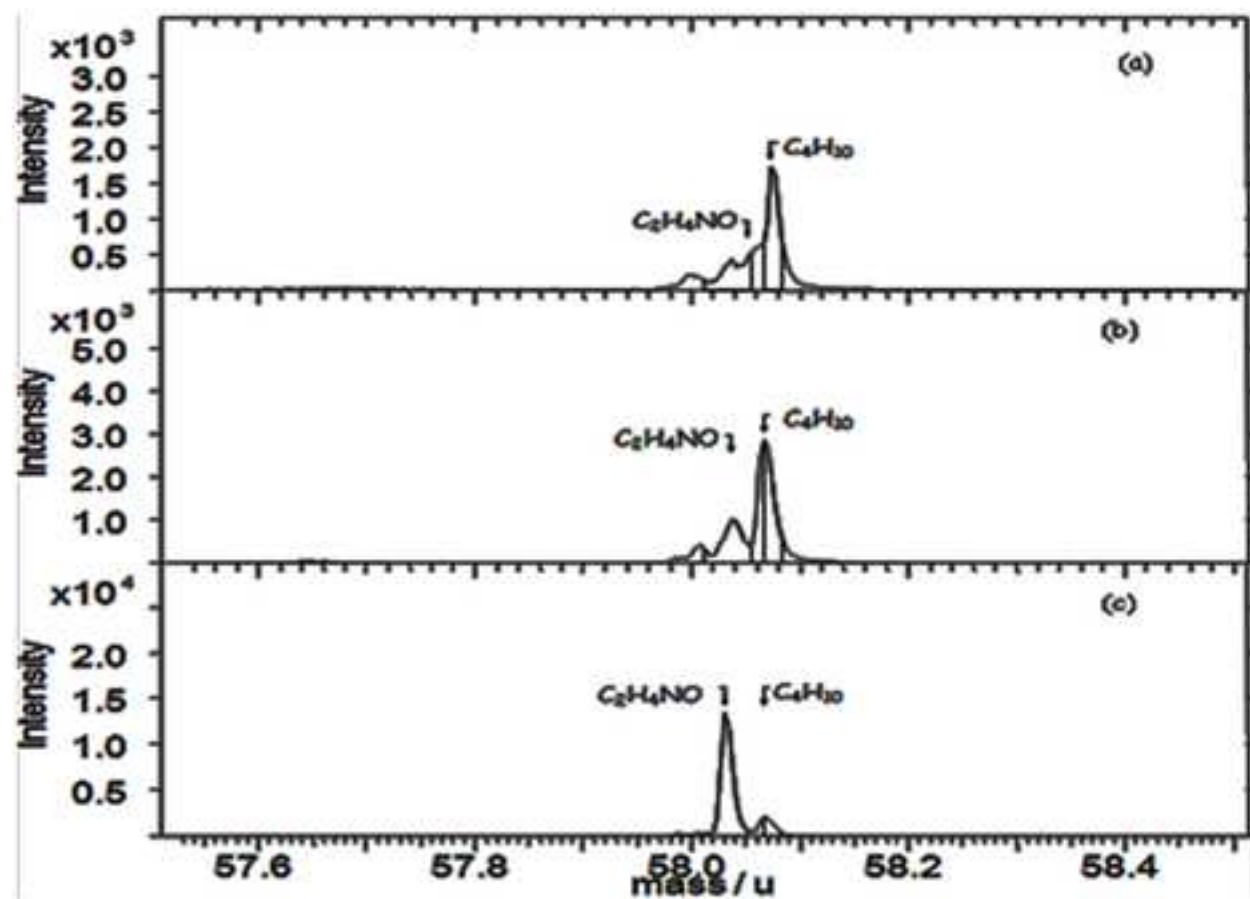
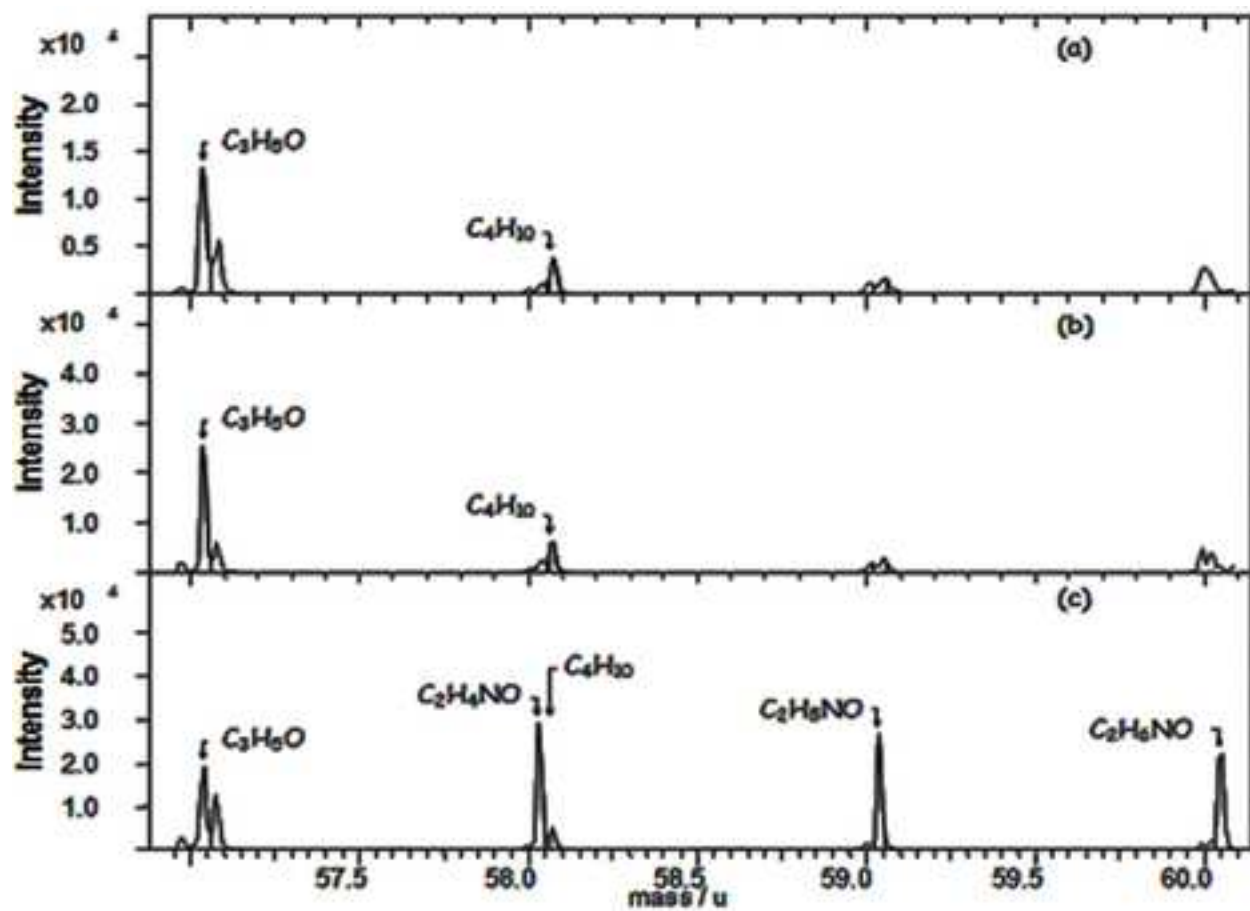
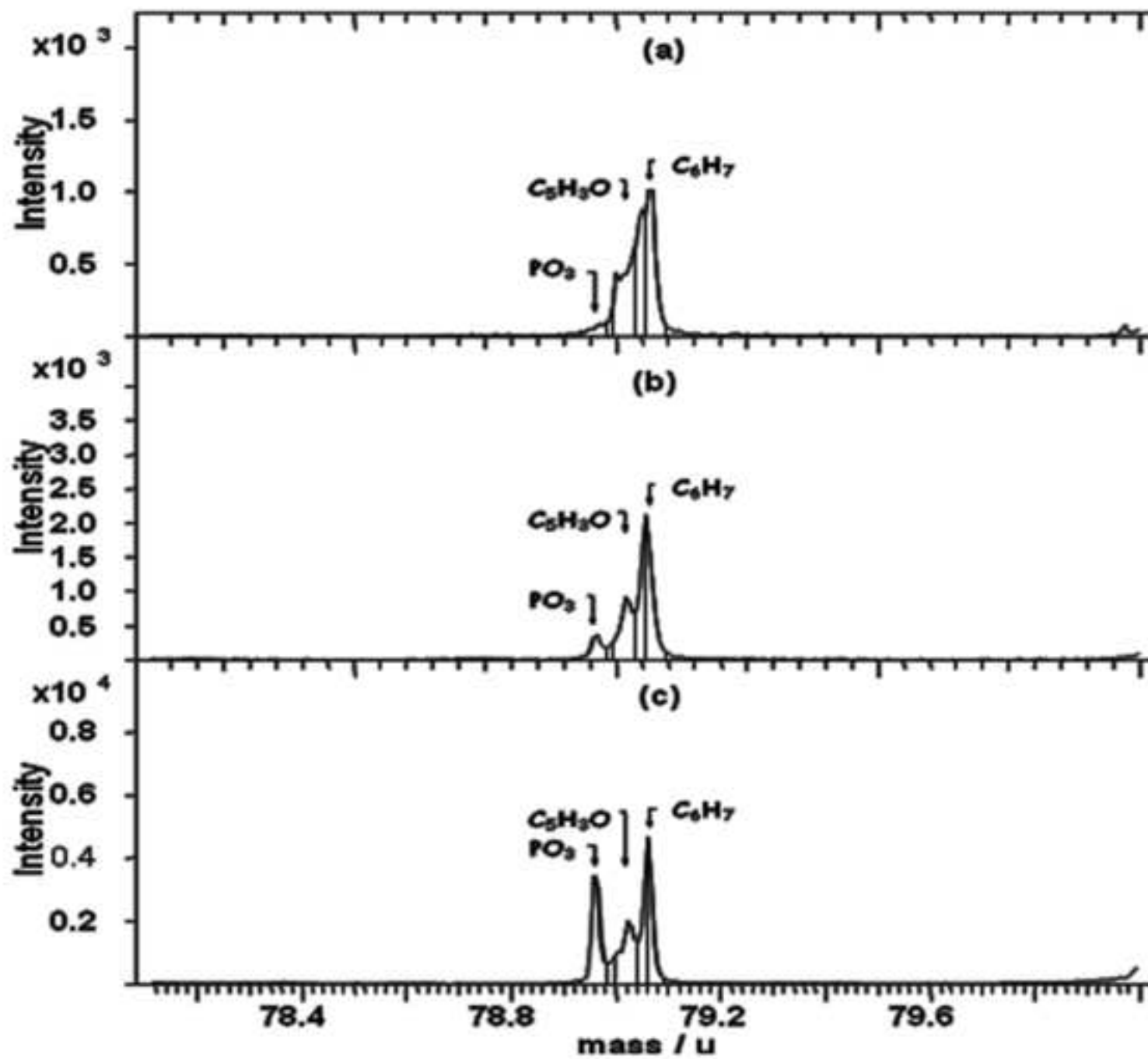
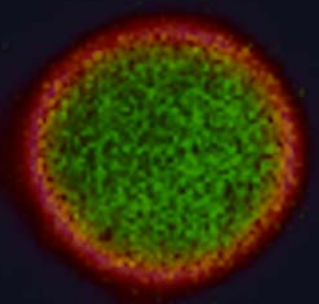
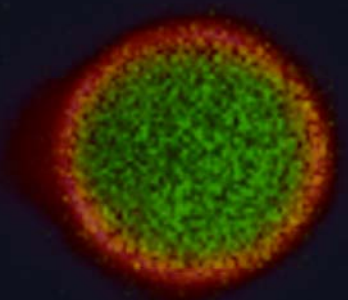


Figure 8. Mass spectra obtained by TOF-SIMS, showing the region  
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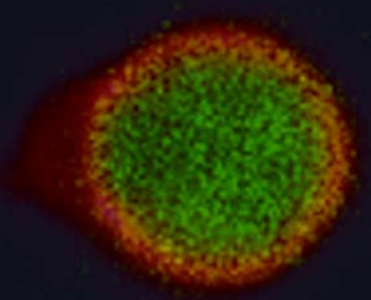




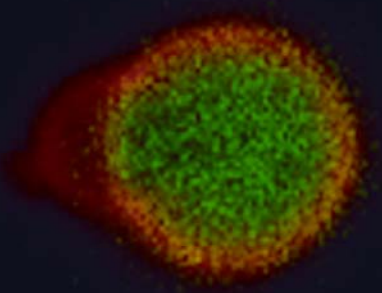
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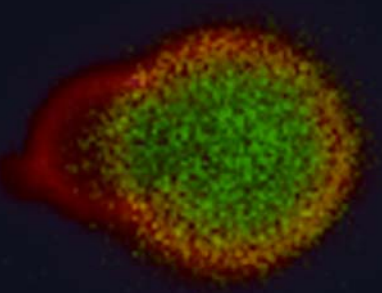
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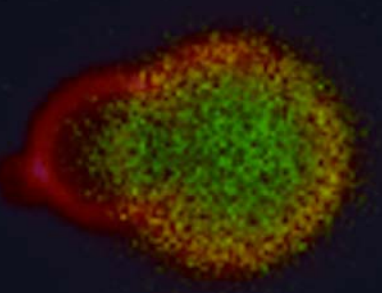
z = 407.034 nm



z = 610.550 nm



z = 814.057 nm



z = 1017.584 nm