

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 ¹³C: 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 μm
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 α -
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[®] 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[®] 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 μ 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 μ 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[®] 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[®] 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 ¹³C 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}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}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 ^1H - ^{13}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[®] 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 ± 2
233 $^{\circ}\text{C}$, resulting in outlet temperature of 111 ± 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[®] 630/650-X₇, 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[®] was obtained by adding a solution of the fluorophore
 239 in dimethyl sulfoxide to a mannitol solution (0.32 µg Bodipy[®]/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[®] LD, equipped with an
 257 Aerodisperser[®] [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, ρ_a : density of air, V_a : velocity of air, V_p : velocity of
264 particle, and ρ_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[®]-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[®] (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[®]), 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° 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 Bi_3^+ beam at 45° 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 ¹³C 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 ¹³C 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 ¹³C 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 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[®]), 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 mm²). 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: ~ 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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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/cm³). 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 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

494 **Acknowledgments**

495 The authors acknowledge funding from the Spanish Government (ISCIII, Acción Estratégica de
496 Salud, PS09/00816), XUNTA de Galicia (PGIDIT, 09CSA022203PR, NANOPULMOGENIC) and
497 IBB/CBME, LA, FEDER/POCI2010. MAEC-AECID fellowship of the Spanish Agency of
498 International Cooperation to S. Al-Qadi is gratefully recognized. Assistance from M. Pastor (NMR
499 service, University Santiago de Compostela) and C. Serra (Centre for Scientific and Technological
500 Support to Research, University of Vigo, E-36310,Vigo,Spain) are highly appreciated.

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}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}C NMR signal is indicated between parentheses.

Chemical Shift (ppm)					
Simple	C1 to C6 (sugar) ^a	CO (acetamide) ^b	CO (glucuronic) ^b	CH ₃ (acetamide) ^c	CO* _d
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 ^a (%)	Feret's diameter ^b (μm)	Real density (g/cm^3)	Aerodynamic diameter (μm)
70/30	3.0	2.3 ± 0.7	1.45 ± 0.06	2.57 ± 0.08
80/20	2.8	2.7 ± 1.3	1.45 ± 0.12	ND
90/10	3.0	2.2 ± 0.4	1.45 ± 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}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[®], (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⁺ 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 C₂H₄NO 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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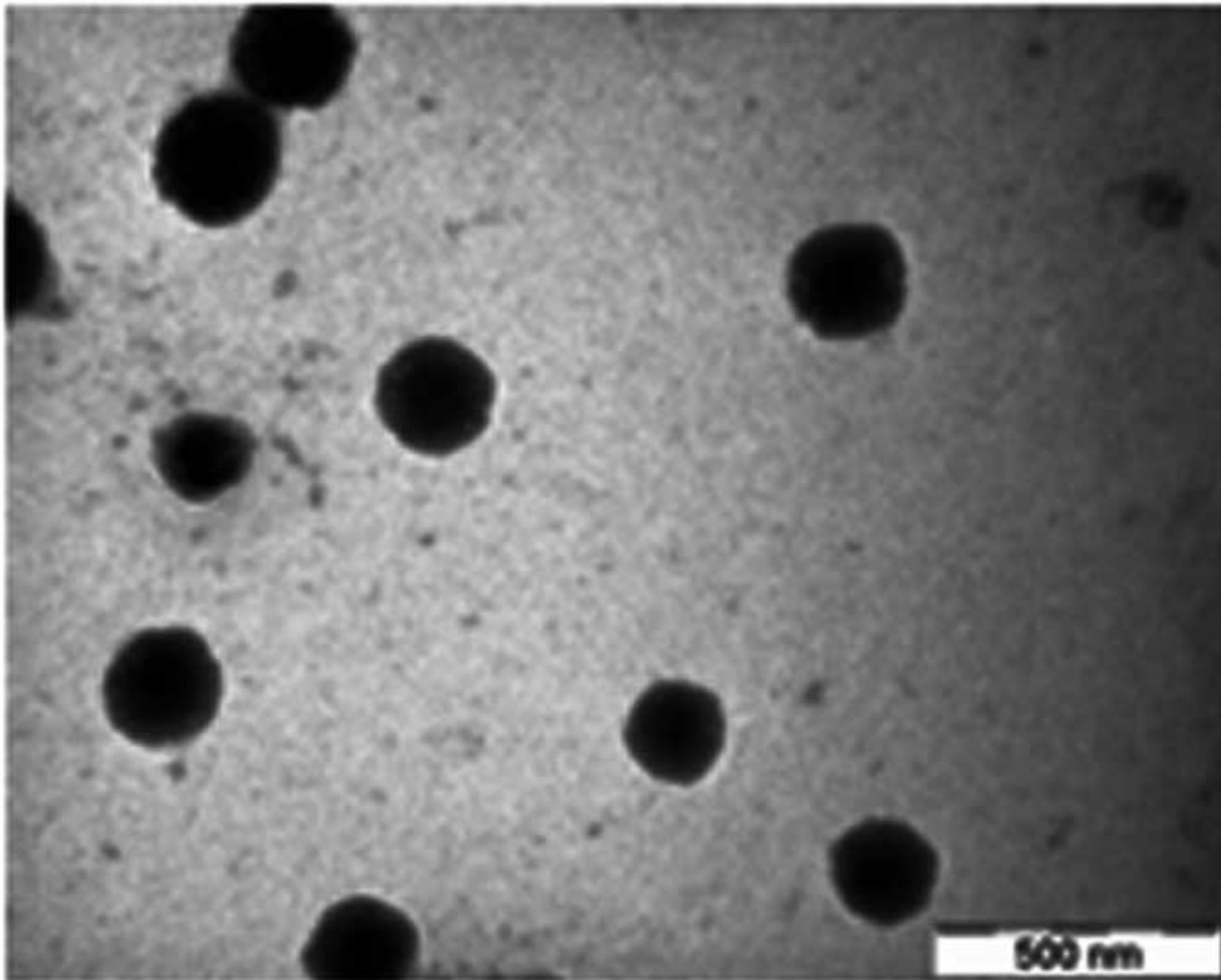


Figure 2. A numbering scheme for hyaluronic acid (above) and ch
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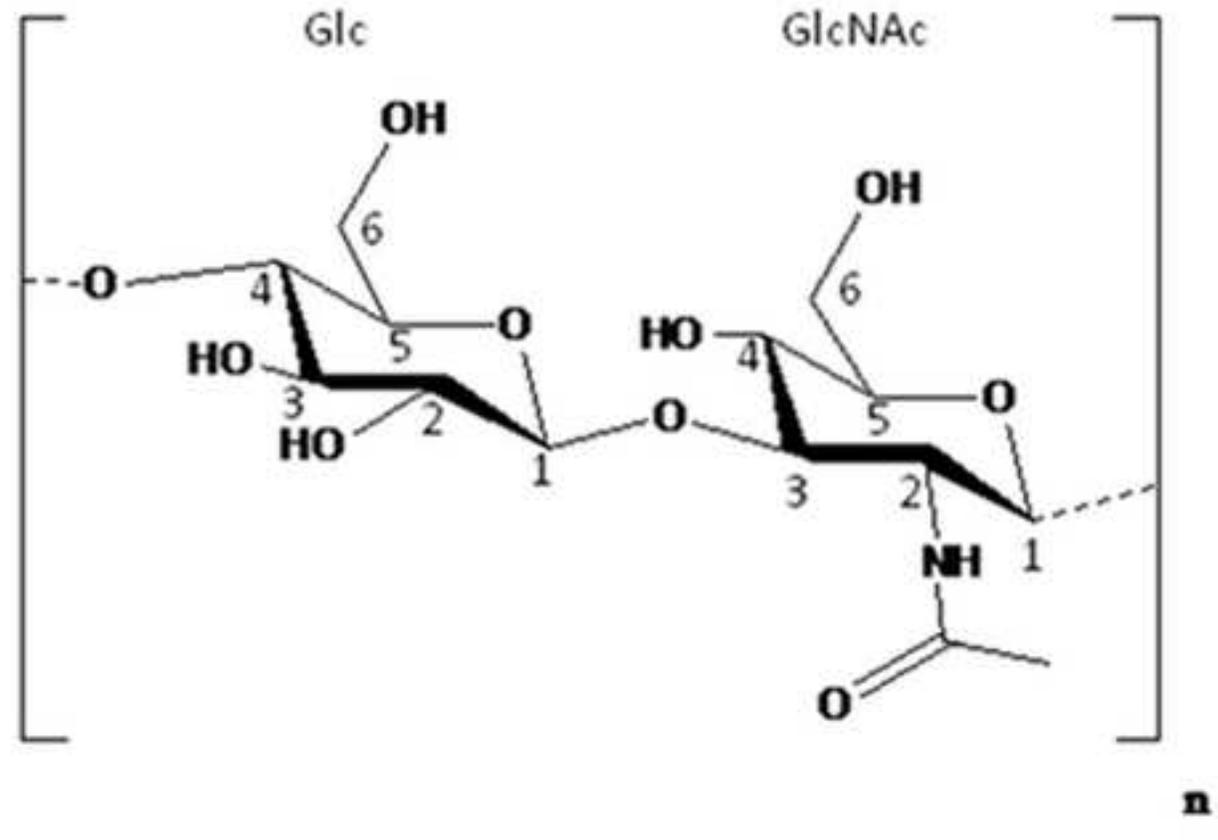
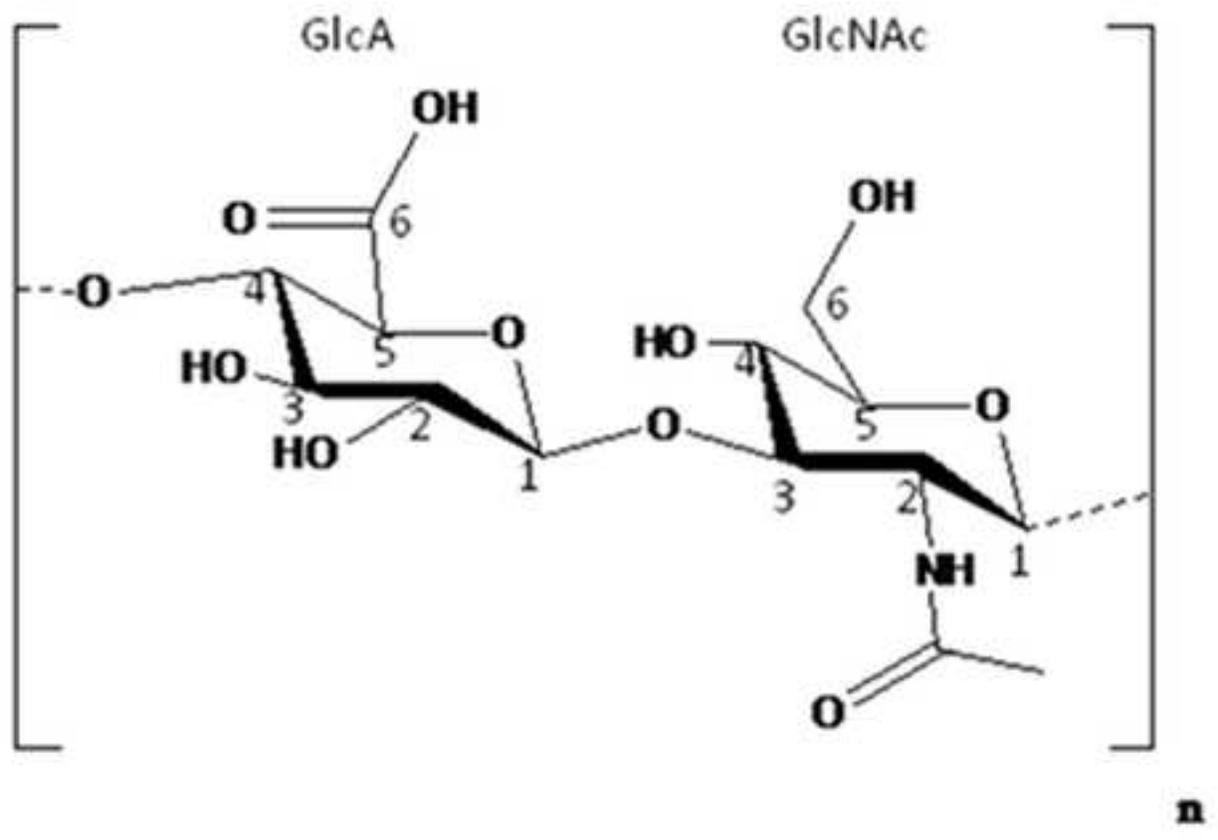


Figure 3. Solid-state ^{13}C -NMR spectra of chitosan/hyaluronic aci
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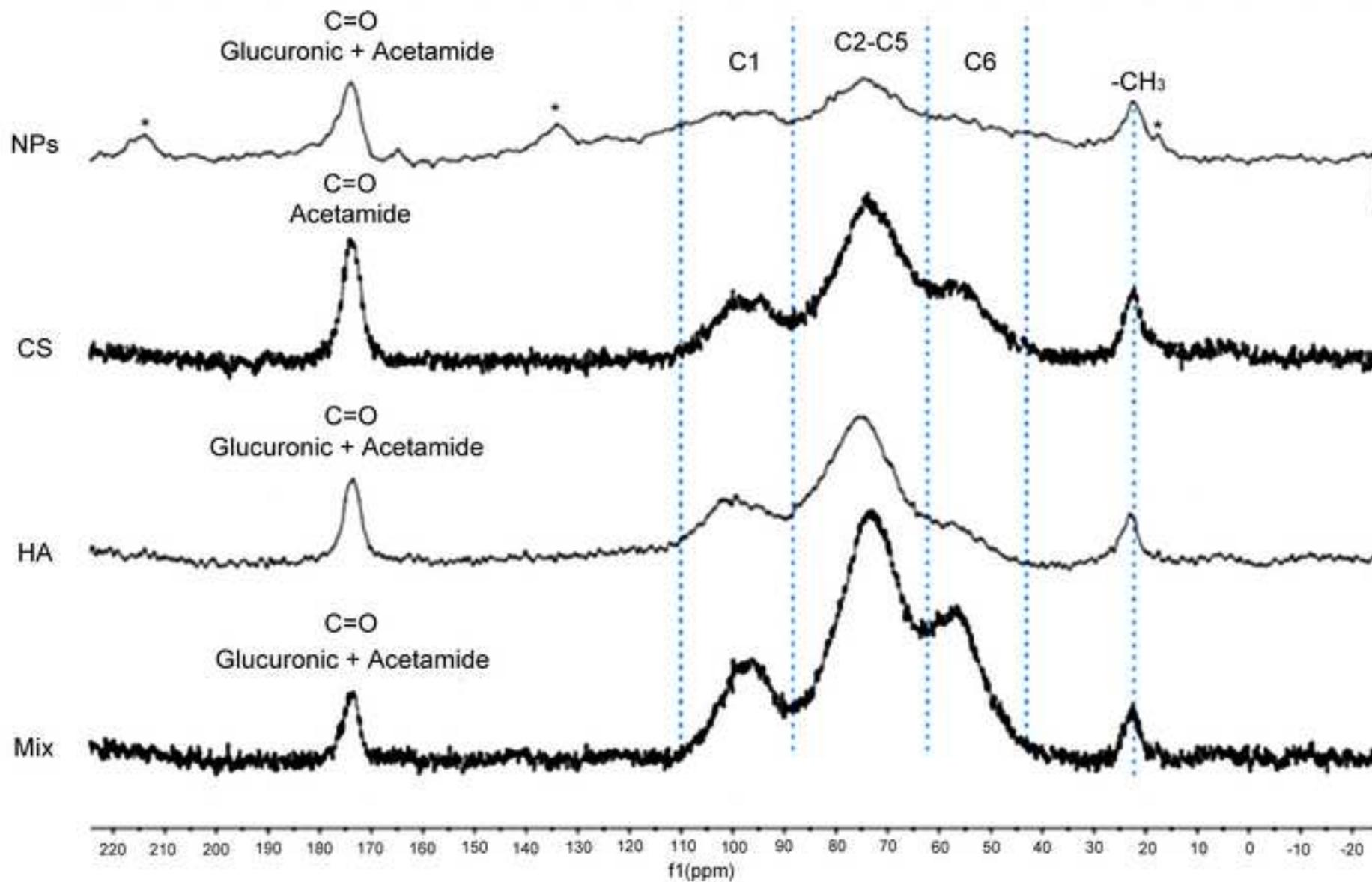


Figure 4. SEM microphotographs of microencapsulated chitosan/hya
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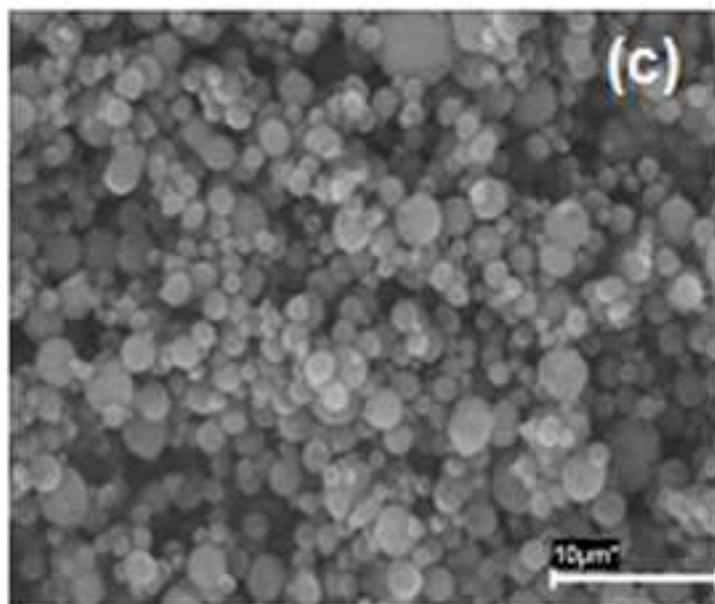
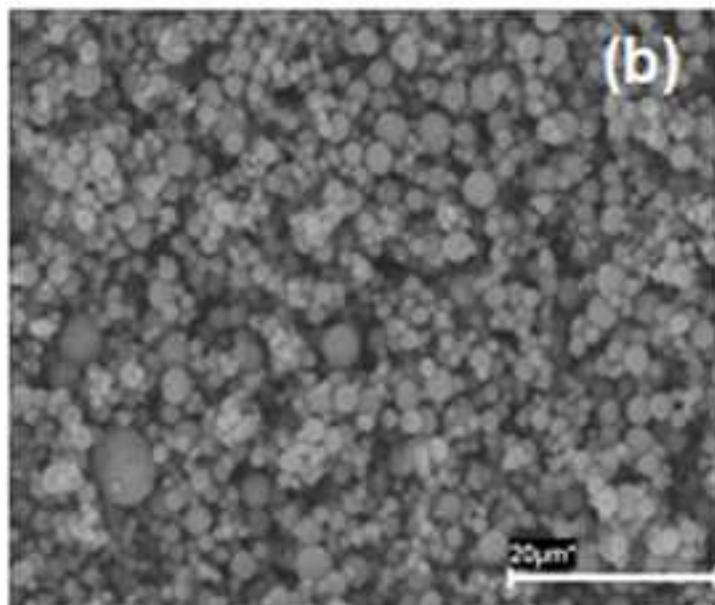
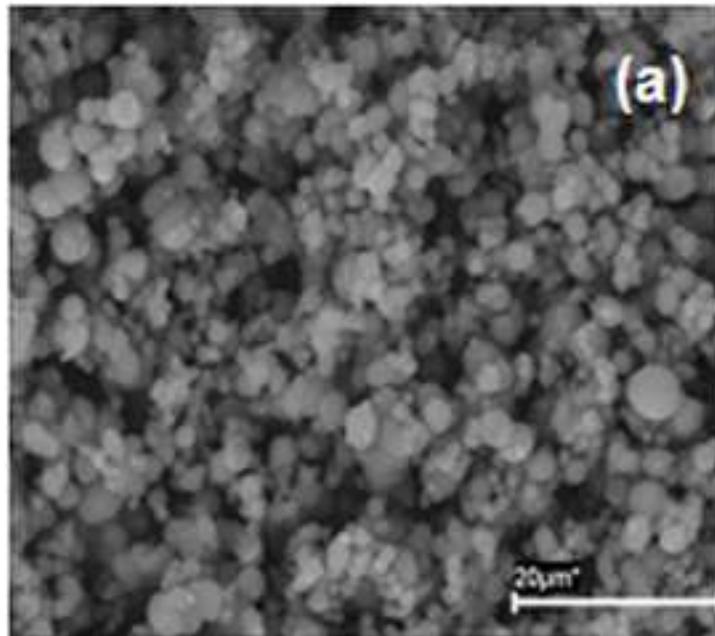


Figure 5. Confocal microscopy images of a mannitol microsphere I
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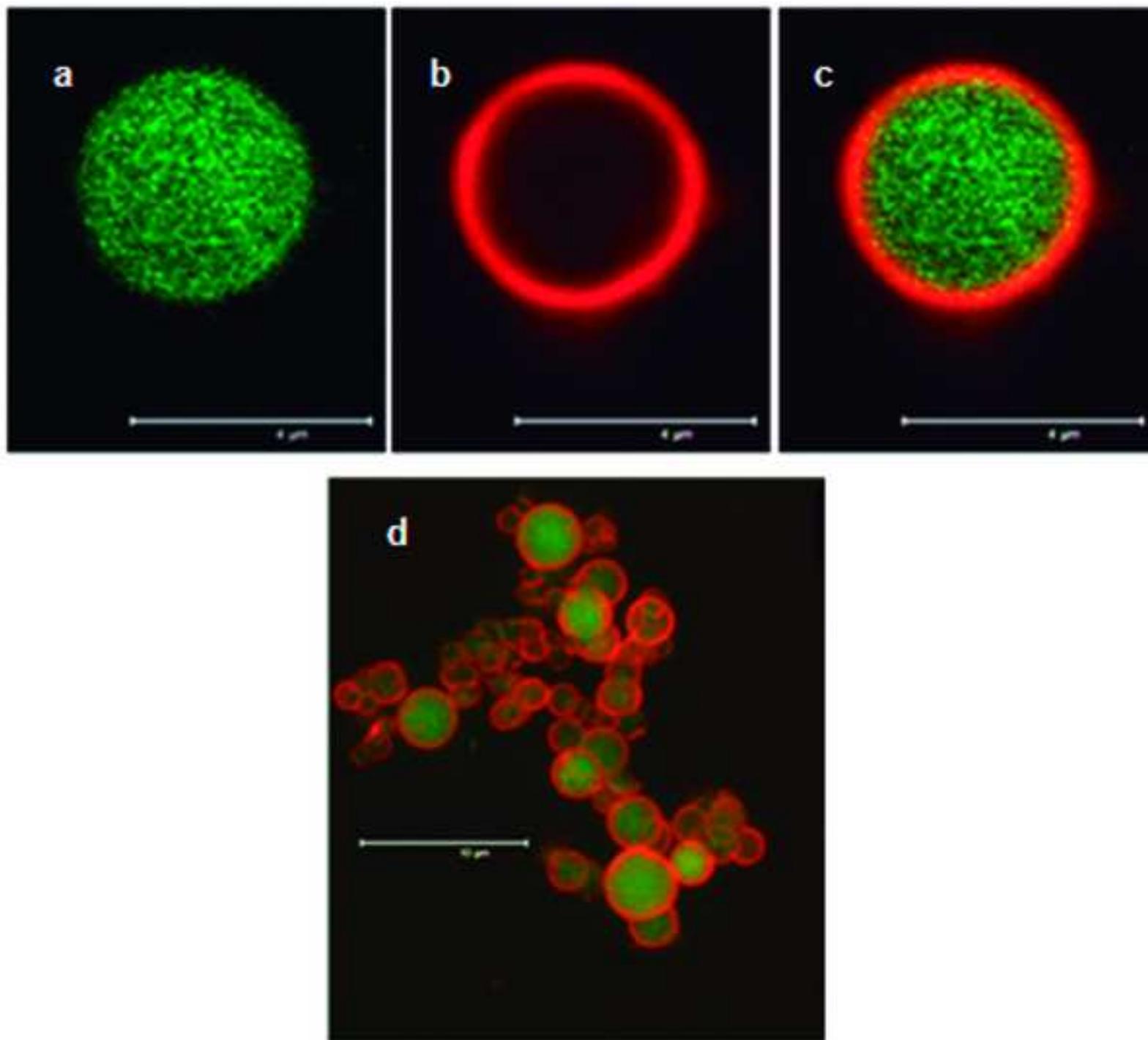


Figure 6. Mass spectra obtained by TOF-SIMS, showing the region
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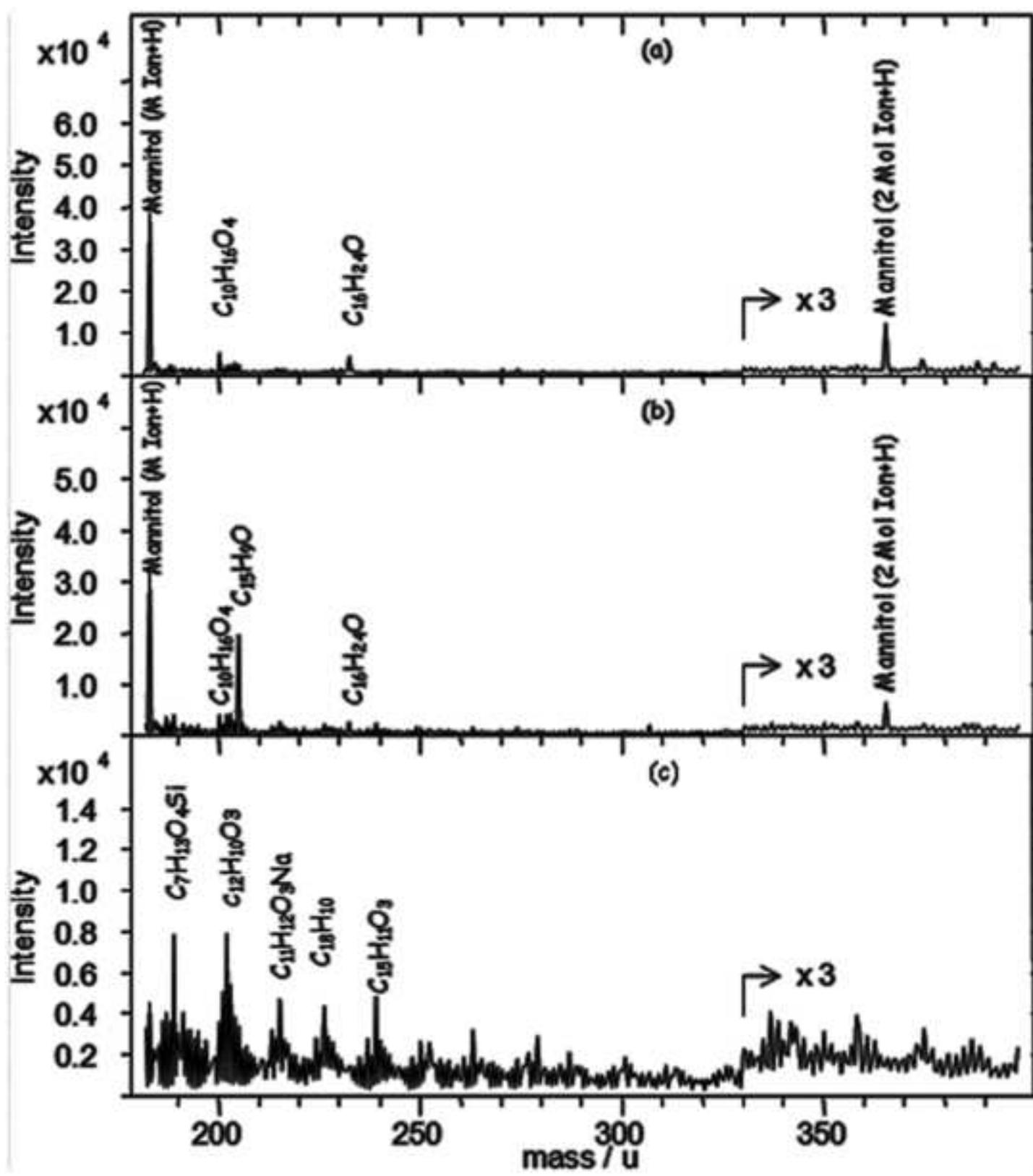
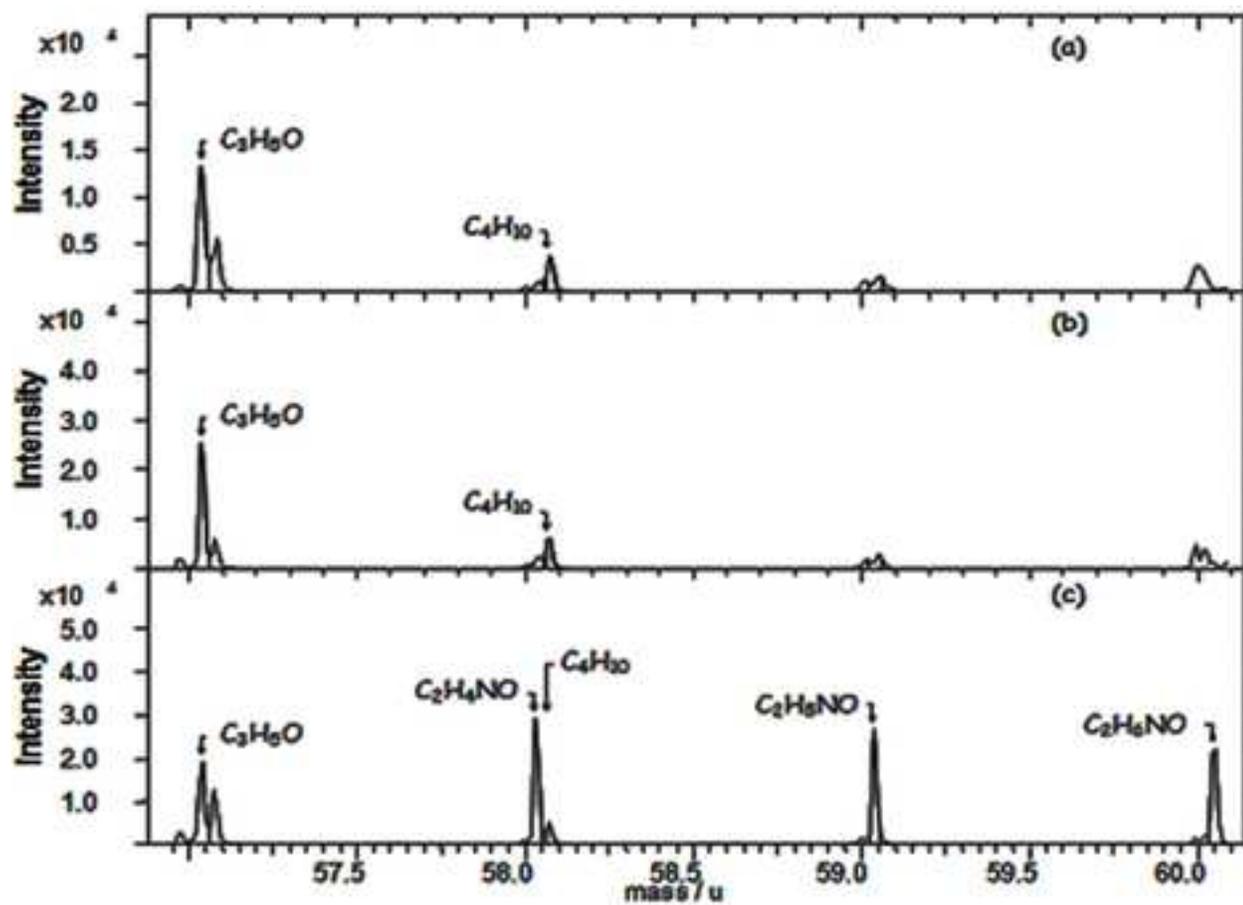
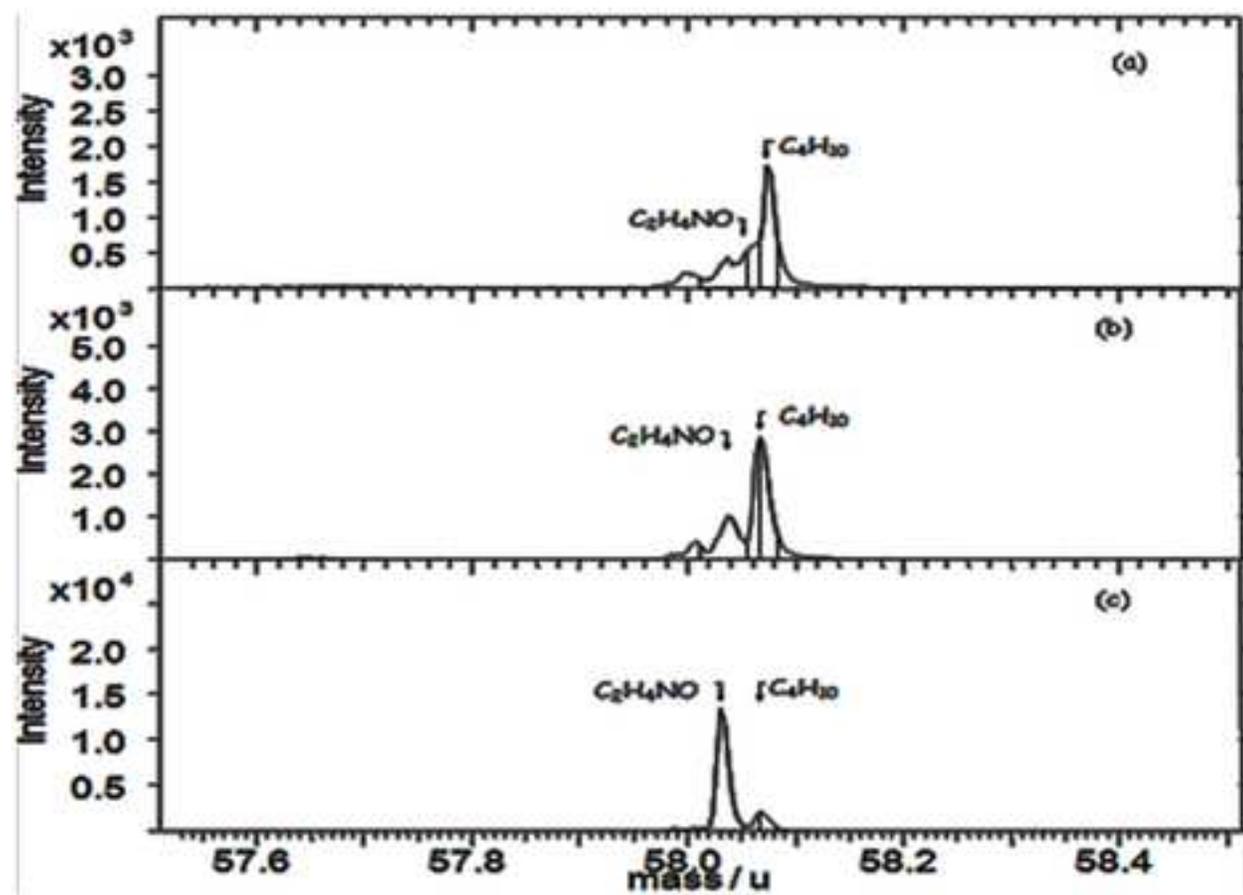


Figure 7. Mass spectra obtained by TOF-SIMS, showing (Fig. 10-1)
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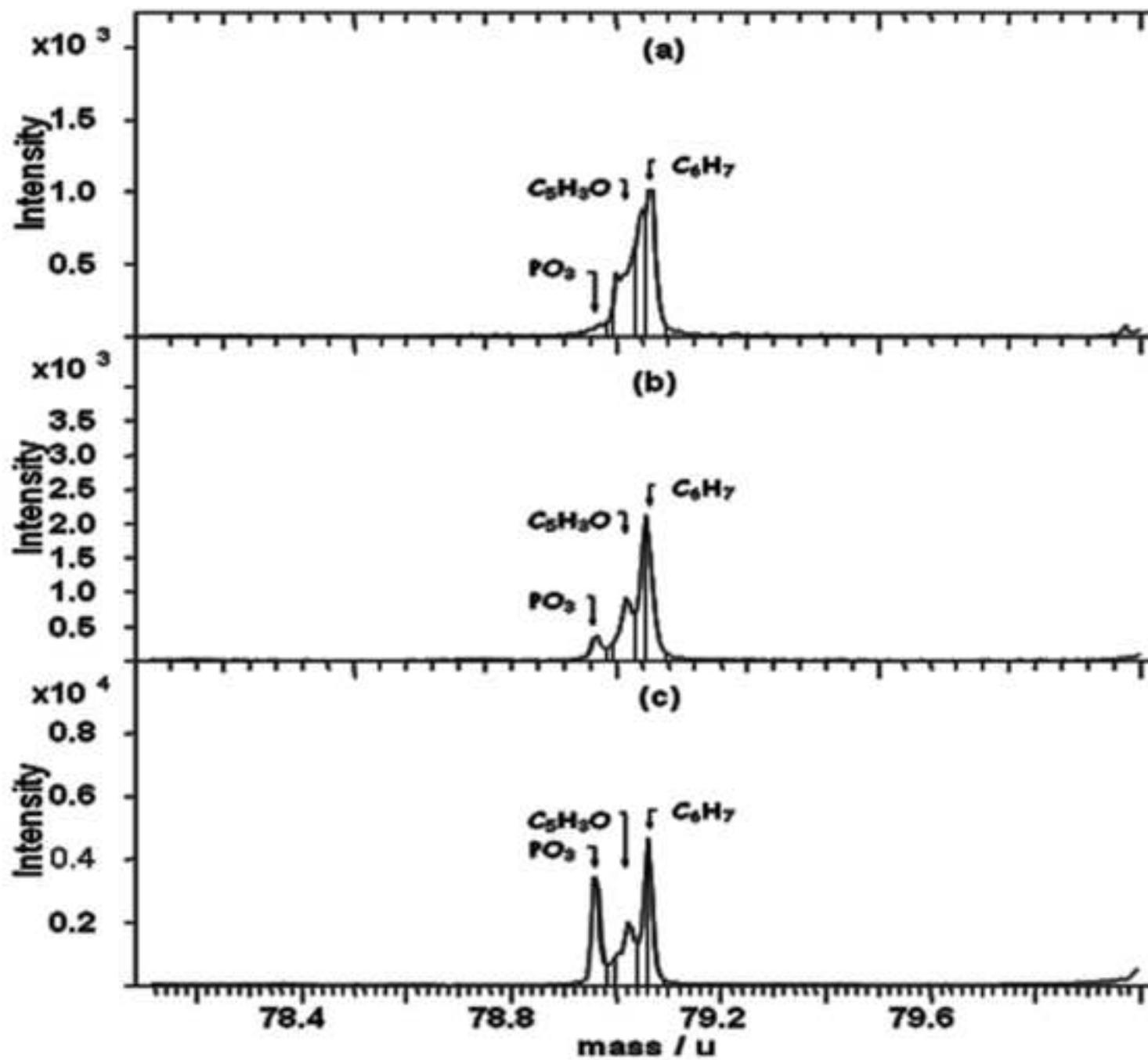


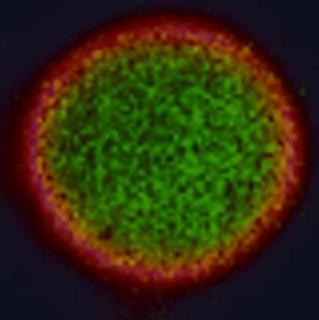
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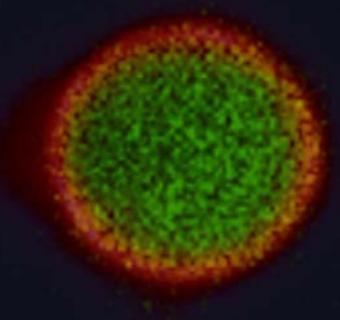
(2)

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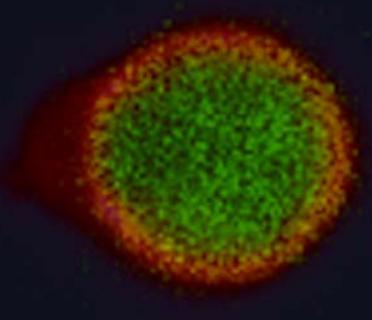




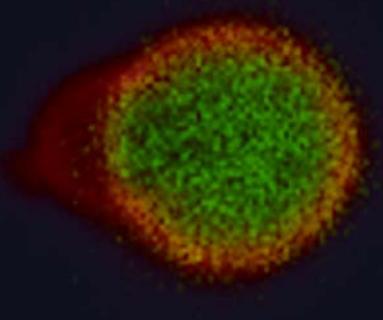
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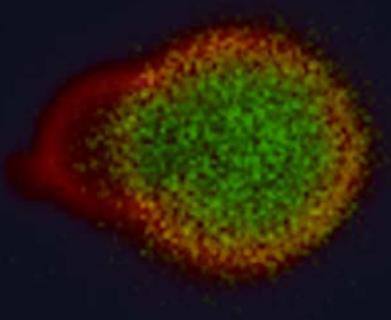
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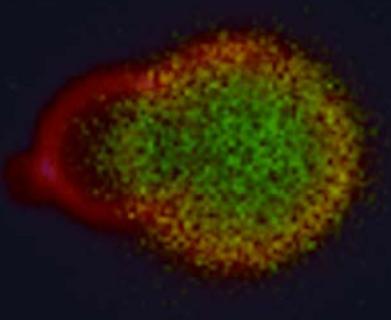
z = 407.034 nm



z = 610.550 nm



z = 814.057 nm



z = 1017.584 nm