

Manuscript Number: FOODHYD-D-12-00246R2

Title: Development of novel ultrathin structures based in amaranth (Amaranthus hypochondriacus) protein isolate through electrospinning

Article Type: Original Research Paper

Keywords: amaranth protein; electrospinning; encapsulation; ultrathin structures

Corresponding Author: Dr. Amparo Lopez-Rubio,

Corresponding Author's Institution: IATA-CSIC

First Author: Marysol Aceituno-Medina

Order of Authors: Marysol Aceituno-Medina; Amparo Lopez-Rubio; Sandra Mendoza; Jose M Lagaron

Abstract: Amaranth protein isolate (API) ultrathin structures have been developed using the electrospinning technique. The effects of pH, type of solvent and surfactant addition on the spinnability, morphology and molecular organization of the obtained structures have been studied. Regarding the effect of pH on API electrospinning, capsule morphologies were only obtained at extreme pH values (i.e. pH 2 and pH 12), which allowed the solubilisation of the proteins, and the process was favoured when the solutions were previously heated to induce protein denaturation. Fibre-like morphologies were only obtained when the solvent used for electrospinning was hexafluoro-2-propanol, as this organic solvent promotes the formation of random coil structures and, thus, an increase in the biopolymer entanglements.

Capsule morphologies were obtained from the API-containing formic acid solutions and this solvent was better for electrospraying than the acetic acid, probably due to the higher viscosity and lower surface tension of the solutions thereof. Addition of 20 wt.-% of Tween 80 considerably improved the formation of capsule-like structures from the formic acid solution, as this surfactant contributed to the formation of alpha helical structures. Similar results were obtained when combining the surfactant with the reducing agent 2-mercaptoethanol. However, denaturation of the protein structure was not sufficient for fibre formation through electrospinning, as the solution properties play a fundamental role in determining the morphology of the electrospun structures.

1 **Development of novel ultrathin structures based in amaranth (*Amaranthus***
2 ***hypochondriacus*) protein isolate through electrospinning**

3

4 Marysol Aceituno-Medina¹, Amparo Lopez-Rubio^{2*}, Sandra Mendoza¹, José María
5 Lagaron²

6

7 ¹ Departamento de Investigación y Posgrado en Alimentos, Facultad de Química,
8 Universidad Autónoma de Querétaro, Cerro de las Campanas s/n, Querétaro, Qro., 76010,
9 México.

10 ² Novel Materials and Nanotechnology Group, IATA-CSIC, Avda. Agustin Escardino 7,
11 46980 Paterna (Valencia), Spain

12

13 *Corresponding author: Tel.: +34 963900022; fax: +34 963636301

14 E-mail address: amparo.lopez@iata.csic.es (A. López-Rubio)

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16

17 **Abstract**

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19 electrospinning technique. The effects of pH, type of solvent and surfactant addition on the
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29 viscosity and lower surface tension of the solutions thereof. Addition of 20 wt.-% of Tween
30 80 considerably improved the formation of capsule-like structures from the formic acid
31 solution, as this surfactant contributed to the formation of alpha helical structures. Similar
32 results were obtained when combining the surfactant with the reducing agent 2-
33 mercaptoethanol. However, denaturation of the protein structure was not sufficient for fibre
34 formation through electrospinning, as the solution properties play a fundamental role in
35 determining the morphology of the electrospun structures.

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40 **Keywords:** amaranth protein, electrospinning, encapsulation, ultrathin structures

41 **1. Introduction**

42 The development of ultrathin and/or nanofibres from biodegradable and biocompatible
43 synthetic and natural polymers through the electrospinning technique has boosted the
44 interest in areas of biomedicine, pharmaceuticals, cosmetics and, more recently, in the food
45 industry due to their potential applications, amongst others, as high-performance
46 encapsulation systems for bioactive compounds (Lopez-Rubio & Lagaron, 2012; Torres-
47 Giner, Martinez-Abad, Ocio, & Lagaron, 2010). Electrospinning is a process that produces
48 continuous polymer fibres with diameters in the submicrometer range through the action of
49 an external electric field imposed on a polymeric solution or melt (Reneker & Chun, 1996).
50 In the food science area, this technique has recently been applied to encapsulate
51 antioxidants (Li, Lim, & Kakuda, 2009; Lopez-Rubio & Lagaron, 2012; Torres-Giner et al.,
52 2010) and probiotic bacteria (Heunis, Botes, & Dicks, 2010; Lopez-Rubio, Sanchez, Sanz,
53 & Lagaron, 2009; Lopez-Rubio, Sanchez, Wilkanowicz, Sanz, & Lagaron, 2012),
54 demonstrating the great potential of electrospinning as a versatile micro- submicro- and
55 nanoencapsulation processing technique to generate ingredients for functional food
56 products. The morphology of the structures obtained through electrospinning can be varied
57 by adjusting the process parameters and, for a certain material, small capsules can be
58 obtained when lowering the polymer concentration and/or increasing the tip-to-collector
59 distance. In this case, the electrospinning process is normally referred to as
60 “electrospraying” due to the non-continuous nature of the structures obtained. The main
61 factors that influence the morphology of the electrospun structures are the solution
62 properties (specifically the viscosity, conductivity and surface tension of the polymer
63 solutions) and the process parameters (mainly voltage, distance to collector, flow rate and
64 ambient humidity) (Chakraborty, Liao, Adler, & Leong, 2009). The solution properties are

65 defined by the polymer type, molecular weight and concentration as well as by the solvent
66 properties. It is generally considered that for fibre development, a critical chain
67 entanglement is needed which usually requires a certain viscosity of the solutions and
68 avoiding too high surface tension values. The size of the fibres of capsules generated can be
69 modified by changing the previous mentioned parameters and, for instance, increasing the
70 polymer concentration and reducing the surface tension or the distance to the collector,
71 leads to greater fibre or capsule diameters (Chakraborty et al., 2009).

72 There is a wide range of polymers which can be used to entrap, coat or encapsulate
73 substances of different types, origins and properties. Recently, the interest to develop
74 functional matrices using natural substances such as proteins, carbohydrates and lipids have
75 increased due to consumer awareness of the environmental damage caused by non-
76 biodegradable materials and health issues. These materials come from renewable resources
77 and they may be nontoxic, edible, and digestible. The importance to develop biodegradable
78 materials is not only focused on food applications, but also on the potential to create a
79 completely new market for commodities or wastes arising from agricultural production. In
80 this context, amaranth (*Amaranthus hypochondriacus*) is a traditional Mexican plant that
81 remains as an underutilized crop which provides both grains and tasty leaves of high
82 nutritional value (Silva-Sánchez *et al.*, 2008). The seed has high protein content (17%), and
83 its amino acid composition is close to the optimum amino acid balance required for the
84 human diet (Schnetzler & Breen, 1994; Teutónico & Knorr, 1985). Contrarily to most
85 common grains, the proteins in amaranth are mainly composed of globulins and albumins,
86 and contain very little or no storage prolamin proteins, which are the main storage proteins
87 in cereals, and also the toxic proteins in celiac disease (Drzewiecki *et al.*, 2003; Gorinstein
88 *et al.*, 2002). Several studies suggest that grain amaranth derivatives represent interesting

89 ingredients for food formulations and promissory materials for the development of edible
90 and/or biodegradable films (Colla, Sobral, & Menegalli, 2006; Elizondo, Sobral, &
91 Menegalli, 2009; Tapia-Blácido, Mauri, Menegalli, & Sobral, 2005). However, to the best
92 of our knowledge, to date there is neither published information about the development of
93 encapsulation systems (nano- or microstructures) based in amaranth protein nor about the
94 processing of this protein by electrospinning.

95 The main objective of this research work was to evaluate the feasibility of producing
96 electrospun structures (fibres and/or beads) from amaranth protein isolate (API) and to
97 evaluate the influence of pH, temperature and the use of different solvents and co-spinning
98 agents (various surfactants and a reducing agent) on the morphology and molecular
99 organization of the electro-deposited material. These structures are envisaged as potential
100 novel fabrication processing morphologies and textures for the protein or as bioactive
101 encapsulation matrices for functional food applications.

102

103 **2. Experimental part**

104 **2.1 Materials**

105 Glacial acetic acid of 99.7% purity and sodium hydroxide pellets of 98% purity were
106 supplied by Panreac (Barcelona, Spain). Formic acid of 95% purity, 1,1,1,3,3,3-hexafluoro-
107 2-propanol (HFIP), 2-mercaptoethanol (2-ME), non-ionic surfactant, polyoxyethylene
108 sorbitan monooleate (Tween 80) and amphoteric surfactant, L- α -phosphatidylcholine were
109 supplied by Sigma-Aldrich. The anionic surfactant, sodium stearyl lactate (SSL) was
110 supplied by Danisco and a commercial amaranth protein concentrate (*Amaranthus*
111 *hypochondriacus* L. Revancha variety) was supplied by Nutrisol (Hidalgo, Mexico).

112

113 **2.2 Preparation of amaranth protein isolate (API)**

114 The amaranth protein isolate (API) used in this study was prepared according to Martínez
115 and Añón (1996). Briefly, the commercial amaranth protein concentrate (APC) was
116 defatted with hexane for 12 h (10% w/v suspension). Then, the amaranth protein
117 concentrate was suspended in water and its pH was adjusted to 9 with a 2 N NaOH
118 solution. The suspension was stirred for 30 min at room temperature and, then, centrifuged
119 20 min at 9000 g. Then, the supernatant was adjusted to pH 5 with 2 N HCl and centrifuged
120 at 9000 g for 20 min at 4°C. The pellet was resuspended in water, neutralized with 0.1 N
121 NaOH and freeze-dried. The protein content was determined by the Kjeldahl technique
122 (AOAC 1996) using a conversion factor of 5.85.

123

124 **2.3 Determination of API molecular weight by gel electrophoresis**

125 The amaranth protein isolate was characterized based on its protein profile using sodium
126 dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The runs were carried
127 out in the following continuous buffer system: 0.5M Tris-HCl pH 6.8/1% (w/v) SDS for the
128 stacking gel, and 1.5M Tris-HCl 8.8/1% (w/v) SDS for the separating gel. The stacking and
129 separating gels were prepared with 4% and 15% (w/v) acrylamide, respectively. Protein
130 samples (10 mg/mL) were dissolved in 0.5 M Tris-HCl, pH 6.8/8% (v/v) glycerol/1% (w/v)
131 SDS/0.05% (w/v) bromophenol blue and centrifuged at 15800g for 5 min; the supernatants
132 (32 µl) were used to load the gel. For runs in reducing conditions the sample buffer
133 contained 5% 2-ME and samples were heated for 1 min in a boiling-water bath. The
134 standard molecular weight proteins (Mark 12™ unstained standard) were supplied by
135 Invitrogen. After electrophoresis, the gel was fixed with trichloroacetic acid (12.5% w/v)
136 for 30 min and stained overnight by addition of Coomassie Brilliant Blue.

137

138 **2.4 Preparation of polymer solutions for electrospinning**

139 The preparation of the different solutions for electrospinning is described below:

140 *2.4.1 Amaranth protein isolate aqueous solutions at different pH values.*

141 Aqueous solutions containing either 1N of glacial acetic acid or 0.01M of NaOH of
142 amaranth protein isolate (5, 10, 12 and 15% w/w) were prepared at different pH values
143 (ranging from 2 to 12) with and without heating (80°C/30 minutes). Before electrospinning,
144 the solutions were stirred until the protein was completely dissolved.

145 *2.4.2 Use of other solvents for the preparation of fibre-forming solutions*

146 Apart from the acetic acid and sodium hydroxide aqueous solutions, two other solvents
147 were tested for dissolving and electrospinning the amaranth protein isolate: HFIP, and
148 formic acid.

149 HFIP solution. Fibre-forming solutions were prepared by dissolving different
150 concentrations of amaranth protein (5, 8, 10 % w/w) in HFIP at 25°C under magnetic
151 stirring until the protein was completely dissolved. The pH of these solutions was around 6.

152 Formic acid solution. Solutions were prepared by dissolving different concentrations of
153 amaranth protein (8, 10, 20 % w/w) in formic acid at 25°C under magnetic stirring until the
154 protein was completely dissolved. The pH of these solutions was around 2.

155 *2.4.3 Effects of different surfactants and a reducing agent on the preparation of fibre-* 156 *forming solutions*

157 Tween 80, Sodium stearoyl lactate (SSL), L- α -phosphatidylcholine, and 2-mercaptoethanol
158 were incorporated in 10, 15, 20, 25% w/w with respect to the weight of the protein in the
159 solution. Each solution was stirred for 1 hour at room temperature before electrospinning.

160

161 **2.5 Characterization of the polymeric solutions**

162 The viscosity of the polymeric solutions was determined using a rotational viscosity meter
163 Visco Basic Plus L from Fungilab S.A. (San Feliu de Llobregat, Spain) using a Low
164 Viscosity Adapter (LCP). The surface tension of the polymer solutions was measured using
165 the Wilhemy plate method in a EasyDyne K20 tensiometer (Krüss GmbH, Hamburg,
166 Germany). Both tests were carried out in triplicate. The conductivity of the solutions was
167 measured using a conductivity meter XS Con6 (Labbox, Barcelona, Spain). The pH values
168 were measured using a multi-parameter analyzer CONSORT C380 from Biotech (Madrid,
169 Spain). All measurements were made at 25°C.

170

171 **2.6 Electrospinning technique**

172 The electrospinning apparatus, a FluidNatek® instrument, trademark of the engineering
173 division of BioInicia S.L. (Valencia, Spain), equipped with a variable high voltage 0–30 kV
174 power supply was used. The anode was attached to a stainless-steel needle with internal
175 diameter 0.9 mm that was connected through a PTFE tubing to the biopolymer solutions
176 kept in a 5 ml plastic syringe. The syringe was disposed horizontally lying on a digitally
177 controlled syringe pump while the needle was vertically directed towards the collector. The
178 needle was connected to the emitting electrode of positive polarity of the high voltage
179 power supply. A positively charged jet of the polymer solution was formed from the Taylor
180 cone that travelled through the air gap and was deposited on the collector. The proprietary
181 amaranth-based electrospun structures (P201131705 Patent Application, 2011) were
182 collected on an aluminum foil sheet attached to a copper grid used as collector. All of the
183 electrospinning experiments were carried out at room temperature in air. The
184 electrospinning environmental conditions were maintained stable at 24°C and 60% RH by

185 having the equipment enclosed in a specific chamber with temperature and humidity
186 control. The electrospinning conditions of voltage, tip to collector distance and feed rate
187 were fixed at 14 kV, 10 cm and 0.3 ml/h, respectively.

188

189 **2.7 Scanning Electron Microscopy (SEM)**

190 The morphology of the electrospun fibres was examined using SEM (Hitachi S-4100) after
191 sputtering the samples with a gold–palladium mixture under vacuum. All SEM experiments
192 were carried out at an accelerating voltage of 10 kV. The diameters of the electrospun
193 structures were measured by means of the Adobe Photoshop 7.0 software from the SEM
194 micrographs in their original magnification. At least, 50 electrospun structures from each
195 sample, were considered to obtain the average diameter.

196

197 **2.8 Optical microscopy**

198 Optical microscopy images were taken using a digital microscopy system (Nikon Eclipse
199 90i) fitted with a 12 V, 100 W halogen lamp and equipped with a digital imaging head. Nis
200 Elements software (Nikon Instruments Inc., Melville, USA) was used for image capturing
201 and the Adobe Photoshop 7.0 software was used for image processing and analysis.

202

203 **2.9 Attenuated total reflectance infrared spectroscopy (ATR-FTIR)**

204 ATR-FTIR spectra were collected in a controlled chamber at 24°C and 40% RH coupling
205 the ATR accessory GoldenGate of Specac Ltd. (Orpington, UK) to a Bruker (Rheinstetten,
206 Germany) FTIR Tensor 37 equipment. All the spectra were collected by averaging 20 scans
207 at 4 cm⁻¹ resolution. Analysis of the spectral data was performed using Grams/AI 7.02
208 (Galactic Industries, Salem, NH, USA) software.

209

210 **2.10 Statistical analysis**

211 One-way analysis of the variance (ANOVA) was performed using XLSTAT-Pro (Win)

212 7.5.3 (Addinsoft, NY) software package. Comparisons between samples were evaluated

213 using the Tukey test ($\alpha= 0.05$).

214

215

216 **3. Results and discussions.**

217 **3.1 Characterization of amaranth protein isolate (API)**

218 The protein content of amaranth protein concentrate (APC) obtained commercially was
219 $30.9 \pm 0.4\%$. After the purification carried out, the amaranth protein isolate (API) obtained
220 had a protein content of $85.5 \pm 0.2\%$. Similar protein contents for API were obtained by
221 Abugoch, Martínez, & Añón (2010) (84.4%) and Martínez & Añón (1996) (80-90%) using
222 the same isolation procedure. According to the results obtained by electrophoresis (cf.
223 Figure 1), the amaranth protein isolate consisted of a mixture of different proteins with
224 molecular weights ranging from ~10 to ~83 kDa. These results are in agreement with the
225 values obtained by other authors (Abugoch *et al.*, 2010; Martínez & Añón, 1996; Marcone,
226 1999).

227

228 INSERT FIGURE 1 ABOUT HERE

229

230

231 **3.2 Effect of pH and temperature on the morphology and molecular organization of**
232 **electrospun API**

233 Initially, the solubility of API at different pH values (ranging from 2 to 12) was
234 investigated. From previous works it is known that the solubility profile of amaranth
235 proteins at various pH values is characterized by a solubility minimum around the mean
236 isoelectric point (between 4.5 and 6.5) reported for different amaranth protein fractions
237 (Konishi, Horikawa, Oku, Azumaya, & Nakatani, 1991), because protein-protein
238 interactions increase as the net electrostatic charges of the molecules are at a minimum and
239 less water interacts with the protein molecules (Salcedo-Chávez, Osuna-Castro, Guevara-

240 Lara, Domínguez-Domínguez, & Paredes-López, 2002). A moderate and high solubility
241 was only obtained at pH 2 and 12, respectively. Consequently, different concentrations of
242 the protein (5, 10, 12 and 15% w/w) were tested at both pH conditions using aqueous
243 solutions containing either acetic acid 1N for pH 2 or sodium hydroxide for pH 12 (0.01M).
244 In the case of the acid solution, protein concentrations greater than 5% w/w, led to the
245 development of a strong gel after 15 minutes of agitation. This could be explained by the
246 formation of hydrogen bonds between the acetic acid solution and the protein amino acids,
247 which induces the polymer chains to arrange in α -helical and β -sheet configurations, both
248 leading to the gelling of the solution (Van der Leeden, Rutten, & Frens, 2000).
249 On the other hand, due to the greater solubility of API in the alkaline solution, it was
250 possible to incorporate up to 15 wt.-% of the protein without gel formation. Both systems,
251 acid and alkaline, were subjected to the electrospinning process. Figure 2 shows SEM
252 images of the structures obtained. Despite systematic variations of applied voltage (10-22
253 kV) and tip-to-collector distance (6-25 cm) no fibre was obtained, but instead, a few
254 capsules together with amorphous material were collected (cf. Figure 2). Amaranth protein
255 isolates are mainly composed by albumins and globulins (Quiroga, Martínez, & Añón,
256 2007; Scilingo, Molina, Martínez, & Añón, 2002), which have a globular structure and
257 their polyelectrolytic character give rise to a multitude of inter- and intramolecular
258 interactions. These characteristics, specifically the strong molecular interactions,
259 complicate the formation of fibre structures. The above-mentioned α -helical and β -sheet
260 polymer chain configurations which may be adopted in the acetic acid solution also keep
261 the rigidity of the chains, preventing the formation of fibres through electrospinning.
262 Table 1 summarizes the morphology and diameters of the structures obtained from API in
263 acetic acid and sodium hydroxide solutions after the electrospinning process. In this table,

264 solution properties (viscosity, conductivity and surface tension) are also included. Failure to
265 obtain electrospun fibres from pure biopolymer dispersions has been previously reported
266 and it is usually an indication of low viscosity and lack of sufficient entanglement (Buchko,
267 Chen, Shen, & Martin, 1999; Wongsasulak, Kit, Mcclements, Yoovidhya, & Weiss, 2007;
268 Wongsasulak, Patapeejumruswong, Weiss, Supaphol, & Yoovidhya, 2010).

269

270

271

INSERT TABLE 1 ABOUT HERE

272

273 The application of heat can help to facilitate denaturation of the proteins, leading to an
274 unfolded state and minimizing the inter- and intra-molecular interactions. Consequently, the
275 developed solutions at the extreme pH conditions, i.e. the one with acetic acid (pH 2, 5%
276 w/w API) and the alkaline solution (pH 12, 15% w/w API), were heated to 80°C, in order
277 to study the influence of heating on the morphology of the electrospun structures. Although
278 temperature did not led to fibre-like morphologies, it facilitated the formation of
279 microparticles or capsules which were deposited on the collector plate from these solutions,
280 probably due to the increased viscosity of the denatured solutions (see Table 1). Some
281 morphological changes could be appreciated between the obtained electrospayed structures
282 and, while dented droplets were obtained from the acid solution, smooth and round
283 capsules were formed from the heated alkaline one. However, the acid solutions seemed to
284 be more efficient for capsule development, as deduced from the collected electrospayed
285 material (cf. Figure 2). The greater facility for capsule development of the API acid
286 solution may be related to the higher viscosity and lower surface tension of this solution,
287 while the greater average capsule size can be explained by its lower conductivity (cf. Table

288 1). On the other hand, because of the oligomeric structure of API proteins, it is possible that
289 high alkaline environments (pH 12) induce their dissociation, thus increasing low molecular
290 weight species (Konishi, Fumita, Ikeda, Okuno, & Fuwa, 1985; Marcone, 1999; Rajendran
291 & Prakash, 1988). This process causes a drop of the viscosity, consequently preventing the
292 electrospinning process (cf. Table 1 and Figure 2). Similar results have been reported by
293 Torres-Giner and co-workers (2008) with alkaline solutions of zein.
294 However, it is worth mentioning that given the dynamic nature of the electrospinning
295 process, the static values obtained for the conductivity, surface tension and viscosity may
296 only serve as a guide. Moreover, working with biopolymers can be challenging, not only
297 due to the property variations depending on source, extraction method and handling
298 procedures, but also due to potential solution property changes with time (Schiffman &
299 Schauer, 2008). It is also known that the rheological behaviour of the solution (specifically
300 the viscoelasticity), more than its viscosity, plays an important role during electrospinning.

301

302

INSERT FIGURE 2 ABOUT HERE

303

304 The infrared spectra of electrosprayed structures obtained using acid acetic as a solvent is
305 shown in Fig. 3. Characteristic peaks for the amaranth protein isolate (API) were identified
306 at $\sim 1634\text{ cm}^{-1}$ and 1533 cm^{-1} , which correspond to the amide I and II regions respectively.
307 The absorption peak at $\sim 1634\text{ cm}^{-1}$ can be attributed to the stretching of the C=O (Amide I)
308 while the peak at 1533 cm^{-1} is due to stretching of C-N and bending of N-H (Amide II).
309 Amide I band has been widely used to study protein folding, unfolding and aggregation
310 with infrared spectroscopy due to its sensitivity to secondary structure of proteins. The
311 spectrum of the API powder (cf. spectrum B in Figure 3) shows bands at 1634 (strong) and

312 1692 cm^{-1} (weak), which are characteristic of β -sheet structures. After electrospinning, the
313 amide I band considerably broadens indicating a greater conformational freedom of the
314 protein chains (Barth, 2007). A previous study with amaranth protein isolates at different
315 pH conditions showed higher quantity of random coils and less structured domains at pH 2
316 (Ventureira et al., 2012), thus confirming the results shown here. Moreover, apart from the
317 broadening, another maximum centred near 1650 cm^{-1} is clearly seen for the electrospayed
318 API structures, which is characteristic of α -helices and unfolded proteins (Barth, 2007).
319 Some residual solvent remained in the electrospayed structures as observed in the infrared
320 spectrum, which was almost eliminated after drying at 60°C during 30 minutes (see arrows
321 in spectra C and D of Figure 3). Comparing these two spectra it is also apparent that upon
322 elimination of the acetic acid, the contribution of the β -sheet configuration to the secondary
323 structure of the protein was clearly diminished (see dotted line in Figure 3 indicating the
324 position of the band related to the β -sheet configuration), which is consistent with a less
325 structured arrangement of the protein chains.

326

327 INSERT FIGURE 3 ABOUT HERE

328

329 Regarding the electrospun structures obtained from the basic solution, no significant
330 spectral differences were observed when compared with the API spectrum (results not
331 shown), just a slight shift of the amide I band from 1634 to 1638 cm^{-1} which may indicate a
332 relaxation of the hydrogen bonds from the secondary structure of the proteins (Barth,
333 2007).

334

335 **3.3 Effect of the type of solvent used during electrospinning**

336 The type of solvent and the physical properties (viscosity, surface tension and conductivity)
337 of the solutions are important parameters in the electrospinning process. In a poor solvent,
338 which is energetically unfavourable, the dissolution of the polymer is an endothermic
339 process and the polymer segments will be attracted to one another in the solution and
340 squeeze out the solvent between them. The polymer chains will adopt a curled
341 configuration with increased polymer-polymer interactions, fact that can lead to gelation.
342 Moreover, the configuration that is adopted by the polymer chains in solution, will affect its
343 intrinsic viscosity. An extended or uncurled configuration of the polymer chain molecules
344 is associated with an increase in the intrinsic viscosity of the solution, while polymer chains
345 that adopt a curled configuration in the solvent will result in an intrinsic viscosity drop. An
346 extended or uncurled configuration of the polymer chains in solution is necessary for fibre
347 formation during electrospinning (Ramakrishna, Fujihara, Teo, Lim, & Ma, 2005).
348 Apart from the acid and basic aqueous solutions mentioned in the previous paragraphs, and
349 with the aim of improving the spinnability of API, two other solvents were evaluated:
350 Hexafluoroisopropanol (HFIP) and formic acid.

351

352 According to the literature, HFIP is a highly versatile and volatile solvent that has been
353 widely used due to its ability to dissolve nylons and proteins and because it provides
354 solutions with excellent physical properties for electrospinning applications (Li, McCann,
355 & Xia, 2005; Matthews, Wnek, Simpson, & Bowling, 2002; Woerdeman *et al.*, 2005).
356 Different protein concentrations (5, 8 and 10% w/w) in HFIP were tested and the
357 morphologies of the obtained electrospun structures are shown in Figure 4.

358

359

INSERT FIGURE 4 ABOUT HERE

360

361 Concentrations of 5% w/w formed beaded fibres, while concentrations of 8% w/w of
362 protein formed flat fibres. Some fibres were also formed from the solution containing 10
363 wt.-% API, but the high viscosity of the same, prevented a stable ejection of a polymer jet
364 from the tip of the syringe. The morphology of the obtained fibres was similar to the one
365 obtained from wheat proteins using the same solvent (Woerdeman *et al.*, 2005). HFIP is a
366 solvent that allows an increase in the percentage of random coil structures, which causes an
367 increase in the hydrodynamic volume and the degree of biopolymer entanglement in
368 solution (Dror *et al.*, 2008; Gupta, Eelkins, Long, & Wilkes, 2005; Hirota, Mizuno, &
369 Goto, 1997). The increase in random coil structures was confirmed by means of FTIR
370 spectroscopy. Figure 5 (cf. spectrum 5E) shows a considerable shift in the position of the
371 amide I band for API from 1634 to 1648 cm^{-1} , which could be attributed to an increase in
372 alpha-helical structures (Yang *et al.*, 2009). The development of this type of conformation
373 in others proteins after electrospinning has been previously reported (Hirota *et al.*, 1997;
374 Stephens, Fahnestock, Farmer, Kiick, & Rabolt, 2005). However, some residual HFIP
375 remains in the fibres (see arrows), fact that severely limits the applications of these
376 structures in the medical, pharmaceutical, and food industries due to the intrinsic toxicity of
377 this solvent. Therefore, alternative non-toxic solvents are desirable to obtain electrospun
378 biopolymer structures (Ki *et al.*, 2005; Li, Cooper, Mauck, & Tuan, 2006).

379

380 INSERT FIGURE 5 ABOUT HERE

381

382 On the other hand, formic acid has been noted as a good organic solvent for various
383 polypeptide-based polymers (Buchko *et al.*, 1999; Ki *et al.*, 2005; Um, Kweon, & Kwang,

384 2003; Wongsasulak *et al.*, 2007; Wongsasulak *et al.*, 2010), and moreover, it is a permitted
385 food additive (Guidance for industry Q3C, 2003). Therefore, the suitability of formic acid
386 for electrospinning of API was also studied. Three different amaranth protein
387 concentrations (8, 10 and 20 % w/w) were evaluated, but the solution containing 20 wt.-%
388 API was very viscous and gelled after some time, preventing the ejection of the polymer jet
389 from the tip of the syringe. Capsule morphologies were obtained from the other two
390 solutions (8 and 10 wt.-% API) as shown in Figure 6.

391

392 INSERT FIGURE 6 ABOUT HERE

393

394 Formic acid was a better solvent than acid acetic for API, but as shown in Figure 6, a
395 fibrillar morphology was not attained with this solvent either. The differences in
396 spinnability and morphology of the obtained structures can be, at least partially explained
397 by the solution properties. Table 2 compiles the conductivity, surface tension and viscosity
398 of the various API solutions in HFIP and formic acid. Comparing the formic and acetic acid
399 solutions (compare Tables 1 and 2), the former shows increased viscosity and lower surface
400 tension. This increase in the viscosity of proteins in formic acid has been previously
401 reported (Van der Leeden *et al.*, 2003) and was attributed to unfolding and swelling of the
402 globular polypeptide structure. The previous authors estimated from intrinsic viscosity
403 measurements of β -lactoglobulin in formic acid that the volume of the polymer was
404 approximately six times greater than that in water. The conformational change was further
405 confirmed by circular dichroism spectroscopy which suggested a substantial increase in the
406 percentage of random coil structures (Van der Leeden *et al.*, 2003). However, only small
407 conformational changes were observed in the electrospayed API structures obtained from

408 the formic acid solution (cf. spectrum C in Figure 5), since just a slight amide I band shift
409 (from 1634 to 1638 cm^{-1}) was observed. According to other studies with amaranth protein
410 isolates, disulfide bonds are involved in their structural stability (Avanza & Añón, 2007;
411 Castellani, Martínez, & Añón, 1999). Therefore, intramolecular disulfide bridges add
412 rigidity and stability to the protein structure, which could prevent the formation of
413 continuous fibres. Moreover, as in the materials obtained from the HFIP solution, some
414 residual solvent was also present in the API structures (see arrows indicating characteristic
415 bands from formic acid).

416 Regarding the surface tension of the API solution in formic acid, it was considerably lower
417 than that of the protein in acetic acid, fact which could have also favoured structure
418 formation through electrospinning, although this surface tension was not low enough for
419 fibre formation (Gupta *et al.*, 2005).

420 Table 2 shows that increasing protein concentration in both solvent solutions leads to a
421 significant increase in both the viscosity and the conductivity of the solutions (the surface
422 tension is mainly dependent on the type of solvent). Moreover, from Table 2 it can also be
423 inferred that too high conductivity values are detrimental for fibre formation. It is known
424 that increased conductivity results in a greater bending instability (Ramakrishna *et al.*,
425 2005), which combined with relatively high surface tension values, only led to capsule or
426 bead formation.

427 From Table 2 it can also be observed that significantly higher surface tension values were
428 obtained for the formic acid solutions in comparison with those obtained with HFIP.

429

430 **3.4 Effect of surfactants and mercaptoethanol addition on the morphology of**
431 **electrospun API structures**

432 In order to improve the spinnability of API in the formic acid solutions, different
433 surfactants were evaluated. It is well-known that a high surface tension of the polymer
434 solution may favour bead formation, whereas electrostatic forces due to charges within the
435 jet have the tendency to elongate and maintain the jet to produce fibres (Ramakrishna *et al.*,
436 2005). The high surface tension of API solutions in formic acid might counteract the
437 electrical forces, thus preventing the successful ejection of a steady polymer jet from the tip
438 of the syringe. In order to reduce the surface tension of the formic acid solution, a food
439 grade nonionic surfactant, Tween 80, an amphoteric surfactant, L- α -phosphatidylcholine
440 and an anionic surfactant, Sodium stearyl lactate (negative charge) were added to the
441 biopolymer solutions. However, L- α -phosphatidylcholine and sodium stearyl lactate
442 (SSL) were not solubilized in the API-containing formic acid solution. This could be
443 attributed to the interactions between the negative charges of the surfactants with the
444 positive charges of the protein, generating insoluble polymer-surfactant complexes that
445 rapidly phase separated from the solution (Kriegel, Kit, McClements, & Weiss, 2009).

446 On the other hand, addition of Tween 80 significantly improved the morphology of the
447 electrospun structures (cf. Figure 7), which appears to be related with the decrease in the
448 conductivity of the solutions (compare results of Table 2 and Table 3). The improved
449 spinnability of polymeric solution by addition of this surfactant has been previously
450 reported by others researchers (Krieger *et al.*, 2009). From Table 3 it can be observed that
451 the viscosity of the solutions was not significantly altered upon addition of Tween 80, and
452 what it is even more surprising the surface tension was slightly increased (fact that could
453 help explaining the smaller size of the capsules obtained).

454

455

INSERT TABLE 3 ABOUT HERE

456

457 On the other hand, conductivity was significantly reduced, which seems to be a key factor
458 for explaining the improved morphology of the electrosprayed materials. The reduction in
459 conductivity could be explained by the binding of surfactant monomers to the backbone of
460 API, thereby reducing its polyelectrolytic character (Kriegel *et al.*, 2009). From Figure 7, it
461 seems that addition of 20 wt.-% of Tween 80 resulted in improved morphologies in
462 comparison with the structures obtained from the protein solution containing just 10 wt.-%.
463 The lower surface tension and conductivity of the former solution could, at least partially
464 explain this result (cf. Table 3).

465

466

INSERT FIGURE 7 ABOUT HERE

467

468 The other strategy attempted to improve the spinnability of the protein solution in formic
469 acid was addition of 2-mercaptoethanol (2-ME), as this substance contributes to
470 denaturation of proteins by reducing disulfide linkages. Figures 5C and 5D show the
471 scanning electron microscopic images obtained using 2-ME and the reducing agent
472 combined with Tween 80, respectively. From the images it seems that the combination of
473 2-ME with the surfactant provided considerably better results, although no fibres were
474 obtained. Figure 8 shows the ATR-FTIR spectra of the structures with 20% of surfactant
475 and with the combination of surfactant and reducing agent. Both the addition of 20% of
476 Tween 80 and Tween 80 with 2-ME led to an increase in alpha helical structures as inferred
477 by the amide I shift from 1634 to 1650 cm^{-1} and 1647 cm^{-1} , respectively (Yang *et al.*, 2009)
478 and, moreover, taking into account that band width is a measure of conformational freedom
479 (Barth, 2007), the structures developed using Tween 80 and with the combination of the

480 surfactant and the reducing agent displayed narrower bands indicating the formation of
481 more rigid structures. Comparing the solution properties using HFIP and formic acid with
482 the additives (Tables 2 and 3) it seems that apart from denaturing the protein structure,
483 lower conductivity and surface tension values are needed for fibre development.

484

485 **4. Conclusions.**

486 Amaranth protein isolate (API)-based ultrathin structures have been developed for the first
487 time using electrospinning. The morphology of the resulting structures was mainly affected
488 by the appropriate choice of solvent and the protein concentration. Fibre morphologies
489 were only obtained using HFIP as the API solvent, while capsule morphologies were
490 developed from formic acid solutions. Addition of Tween 80 and a combination of this
491 surfactant with the reducing agent 2-mercaptoethanol resulted in improved morphology of
492 the encapsulates and to enhanced spinnability of the API-containing formic acid solutions.
493 This study has also demonstrated that the ability to generate encapsulation structures from
494 API depends, not only on the protein conformation, but also on the solution properties
495 (conductivity, surface tension and viscosity). The API structures generated from formic
496 acid solutions could find applications as new food ingredients or as encapsulation structures
497 for food ingredients.

498

499

500 **5. Acknowledgements.**

501 The authors acknowledge Dr. Benito Manrique de Lara y Soria for providing the amaranth
502 protein concentrate used in this study. A. Lopez-Rubio is recipient of a Ramon y Cajal
503 contract from the Spanish Ministry of Science and Innovation. The authors thank the

504 Spanish MICINN projects MAT2009-14533-C02-01, FUN-C-FOOD (CSD2007-00063),
505 and Mexican project FOMIX-QRO-2011-C02-175350 for financial support and Mexican
506 National Council for Science and Technology (CONACYT) for a graduate fellowship, to
507 author Marysol Aceituno-Medina.

508

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Figure captions

Figure 1. Electrophoretic profile of amaranth protein isolate (API): a) standards; b) SDS-PAGE with 2-ME; c) SDS-PAGE without 2-ME.

Figure 2. SEM (a, c, e, g) and optical microscope (b, d, f, h) images of API electrospun structures from acetic acid (50/50 %v/v) and sodium hydroxide (0.01M) solutions at different pH: pH 2 (a, b); pH 2 heating (c, d); pH 12 (e, f); pH 12 heating (g, h). Scale bar: 5 μm .

Figure 3. ATR-FTIR spectra of: (A) Acetic acid; (B) Amaranth protein isolate (API); (C) Electrospayed API from acetic acid solution; (D) Same as (C) after drying at 60°C during 30 minutes. Dotted line indicates the position of the band related to β -sheet structures. Arrows point out to characteristic bands arising from the presence of solvent in the structures. Spectra have been offset for clarity.

Figure 4. SEM images of electrospun amaranth fibers obtained from HFIP solutions at different protein concentrations: a) 5%; b) 8%; c) 10% w/w. Scale bar: 20 μm .

Figure 5. ATR-FTIR spectra of: (A) Amaranth protein isolate (API); (B) Formic acid; (C) Electrospayed API from formic acid solution; (D) HFIP; (E) Electrospun API structures from HFIP solution. Dotted line indicates the position of the band related to β -sheet structures present in API. Arrows point out to characteristic bands arising from the presence of solvent in the structures. Spectra have been offset for clarity.

Figure 6. SEM images of electrospun amaranth structures obtained from formic acid solutions at different protein concentrations: a) 8%; b) 10%. Scale bar: 5 μm .

Figure 7. SEM images of amaranth structures obtained from formic acid solutions containing: a) 10% Tween80; b) 20% Tween80; c) 20% 2-mercapthoethanol; d) 15% Tween 80 + 20% 2-mercapthoethanol.

Figure 8. ATR-FTIR spectra of: (A) Amaranth protein isolate (API); (B) Tween80; (C) Electrospayed API from formic acid solution containing 20% Tween80; (D) Electrospayed API from formic acid solution containing 15% Tween80 + 20% 2-mercaptoethanol. Dotted line indicates the position of the band related to β -sheet structures present in API. Spectra have been offset for clarity.

Figure 1
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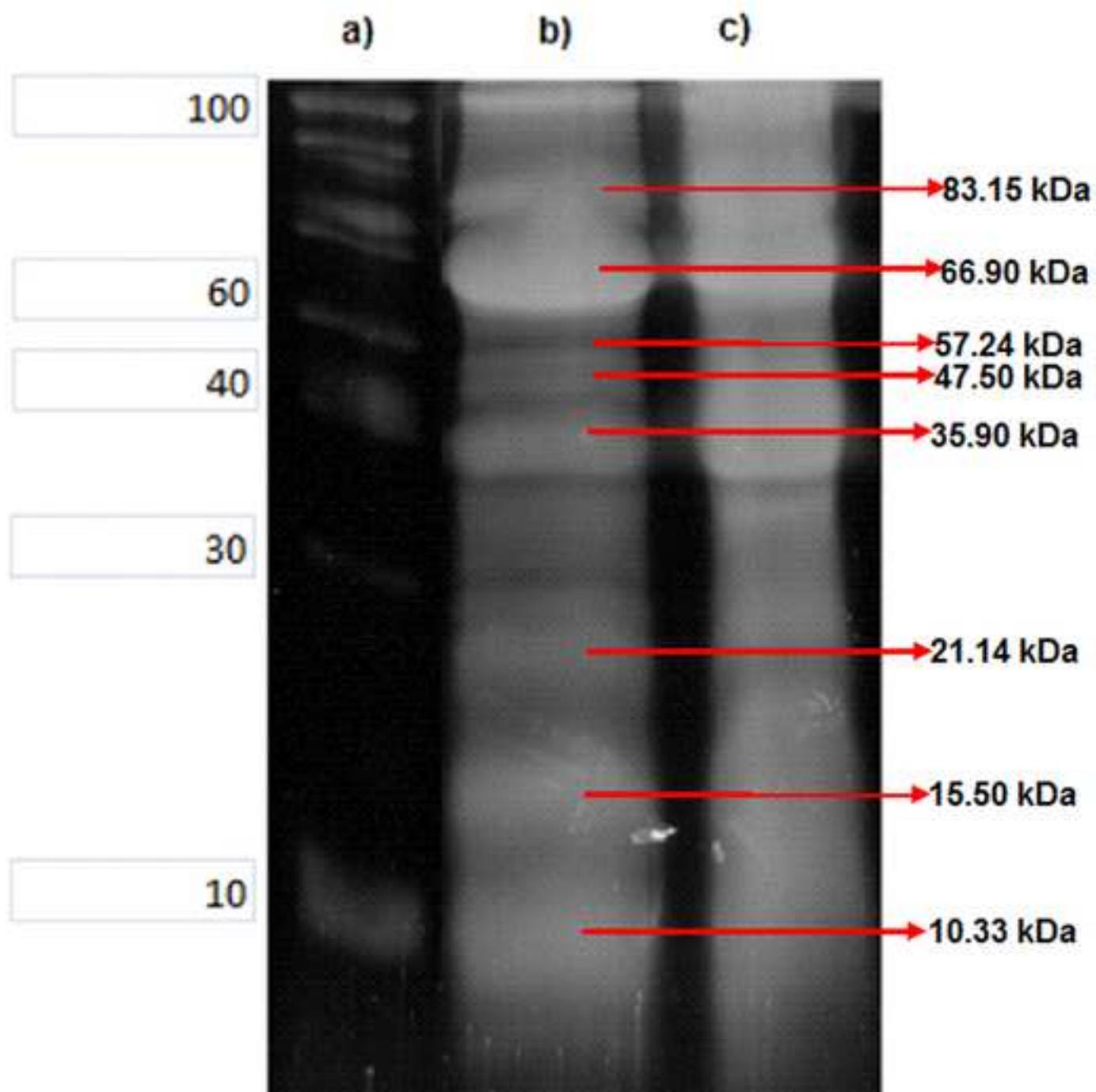


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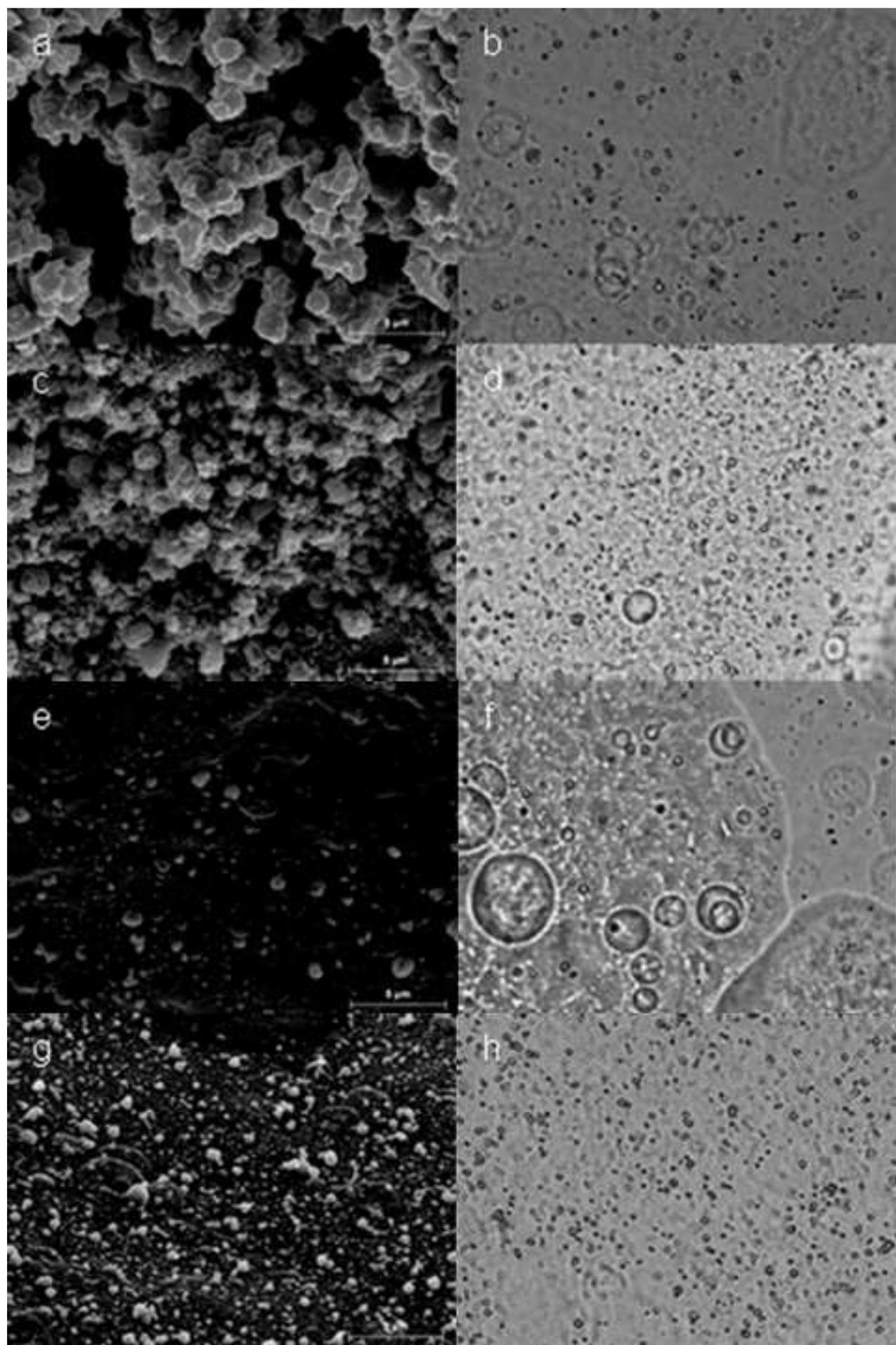


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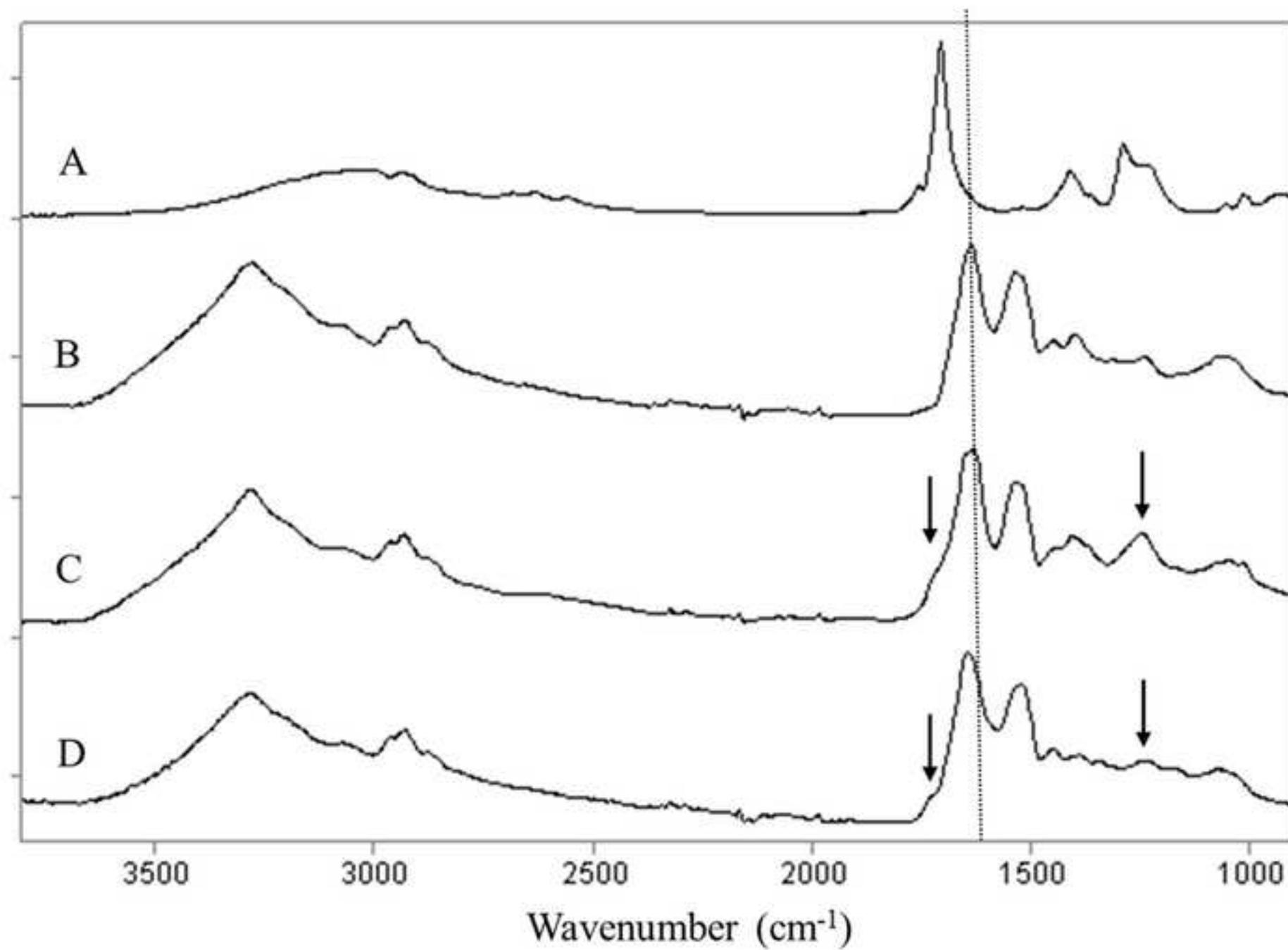


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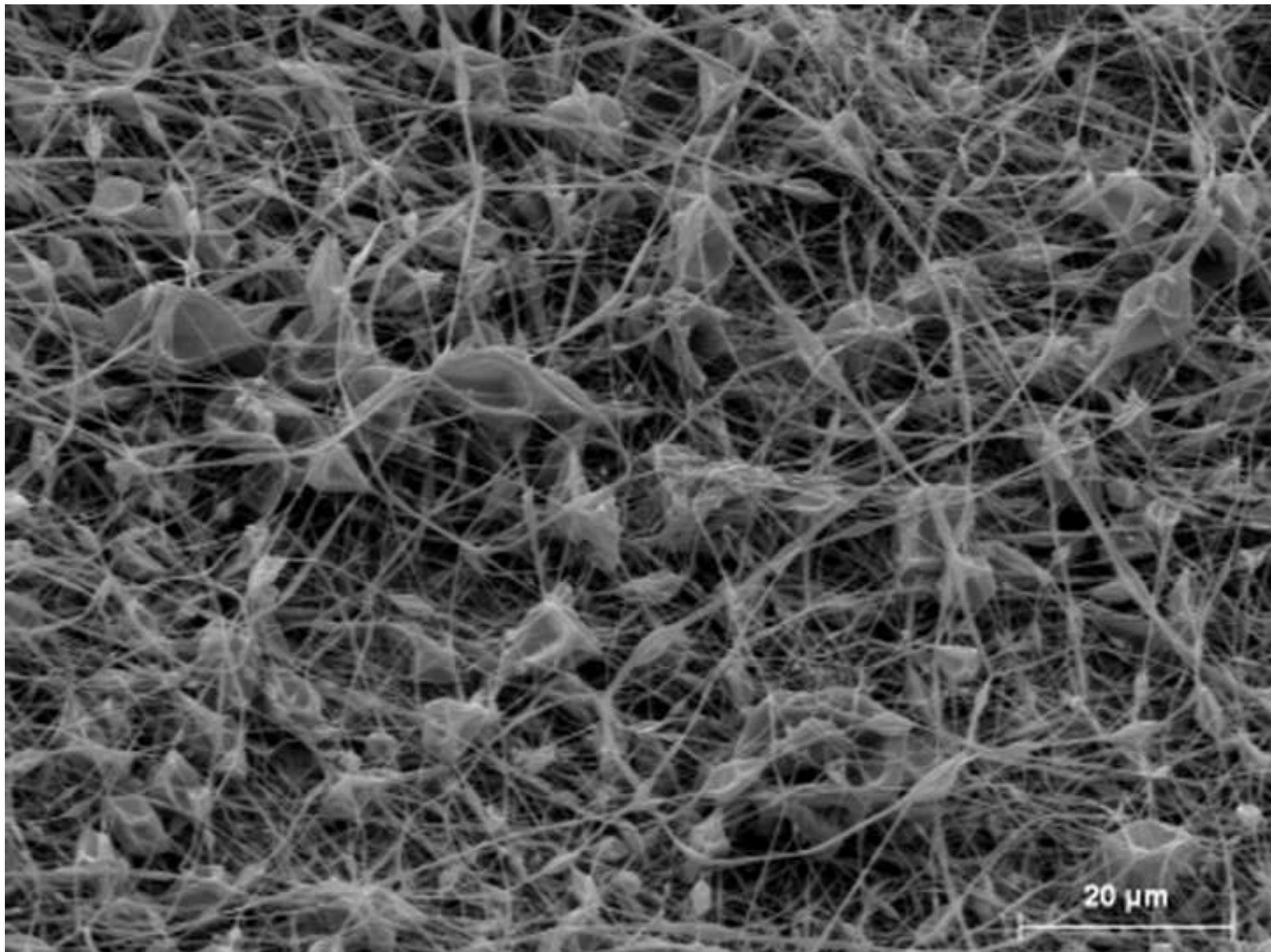


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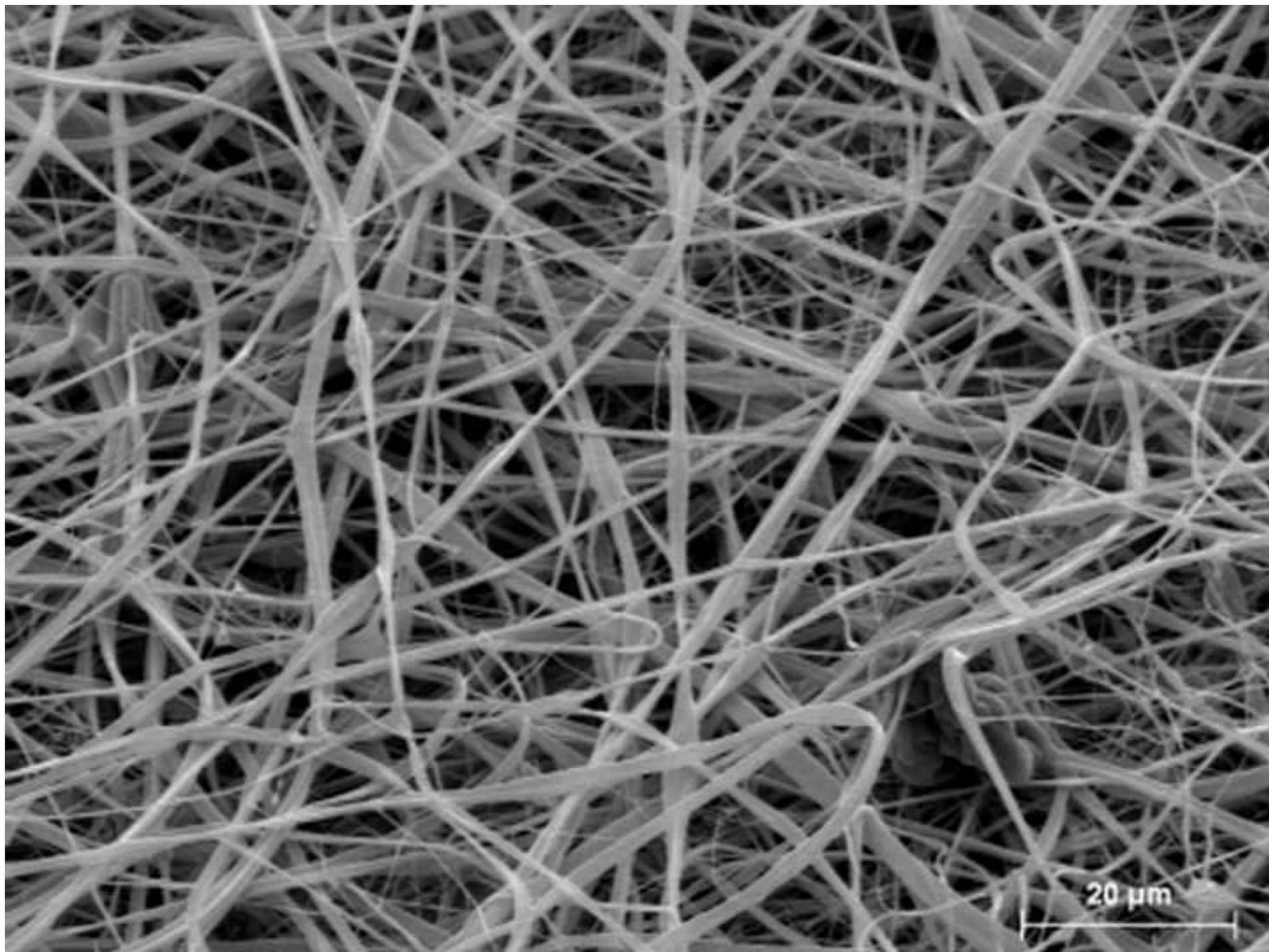


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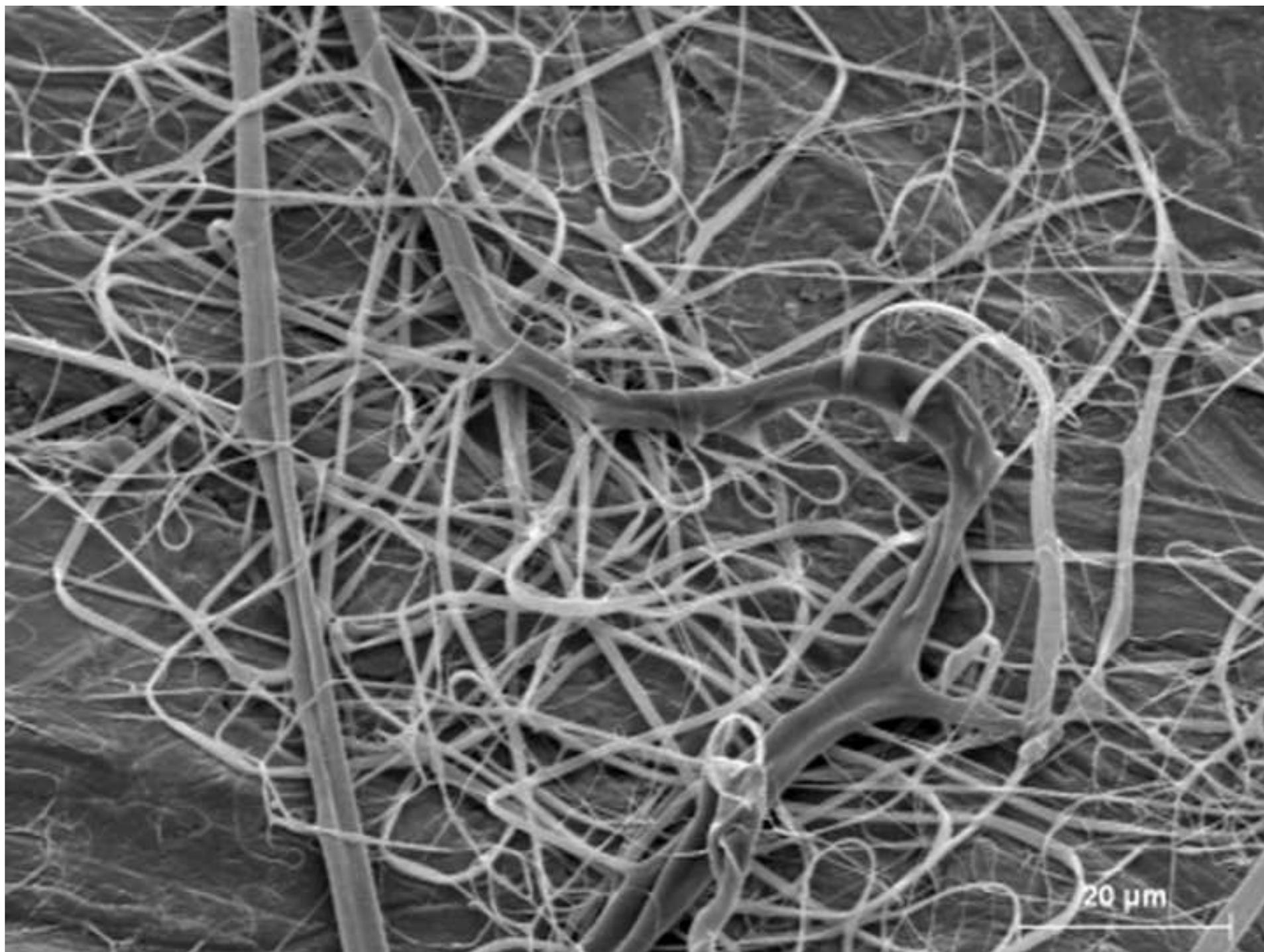


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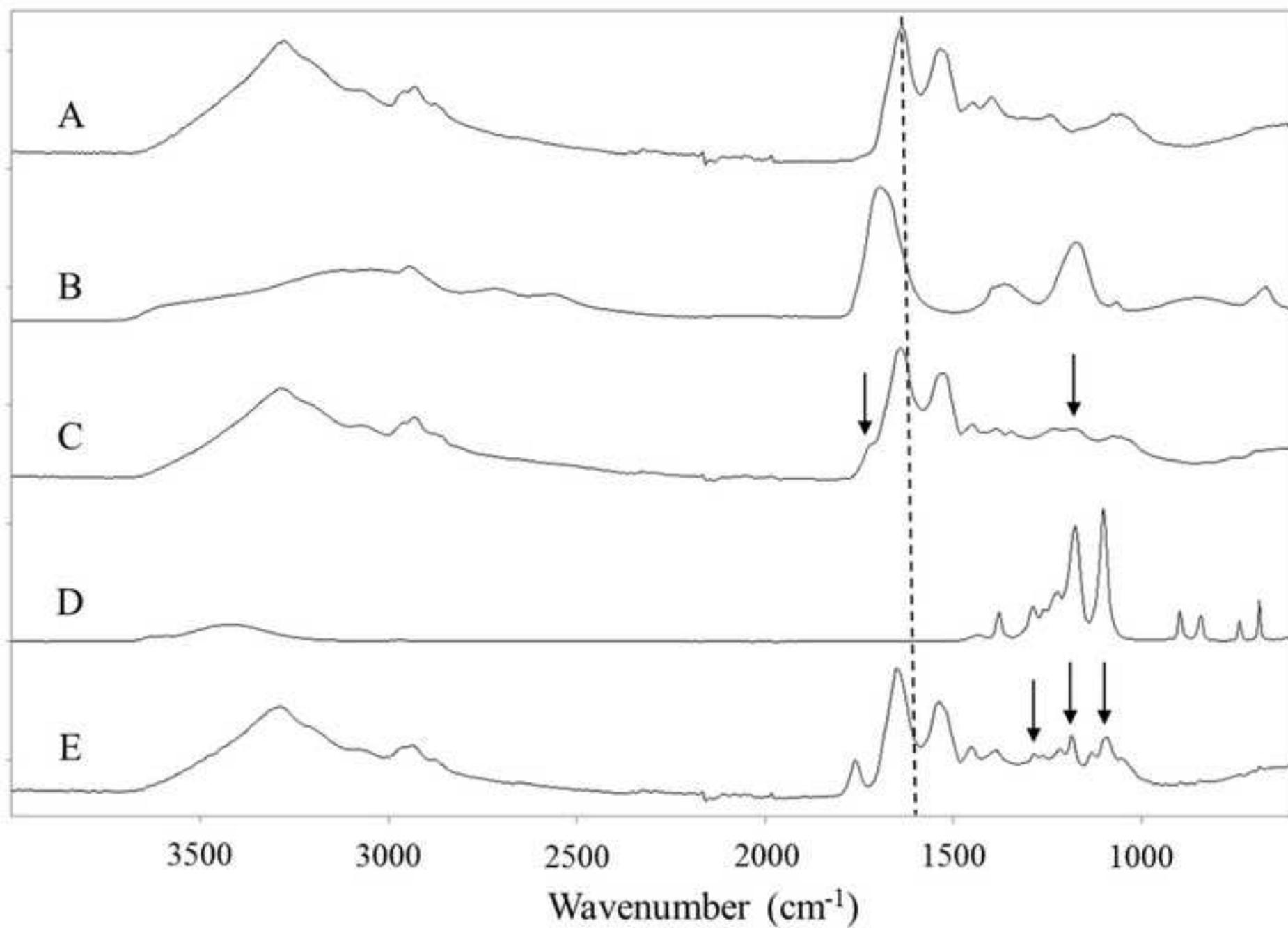


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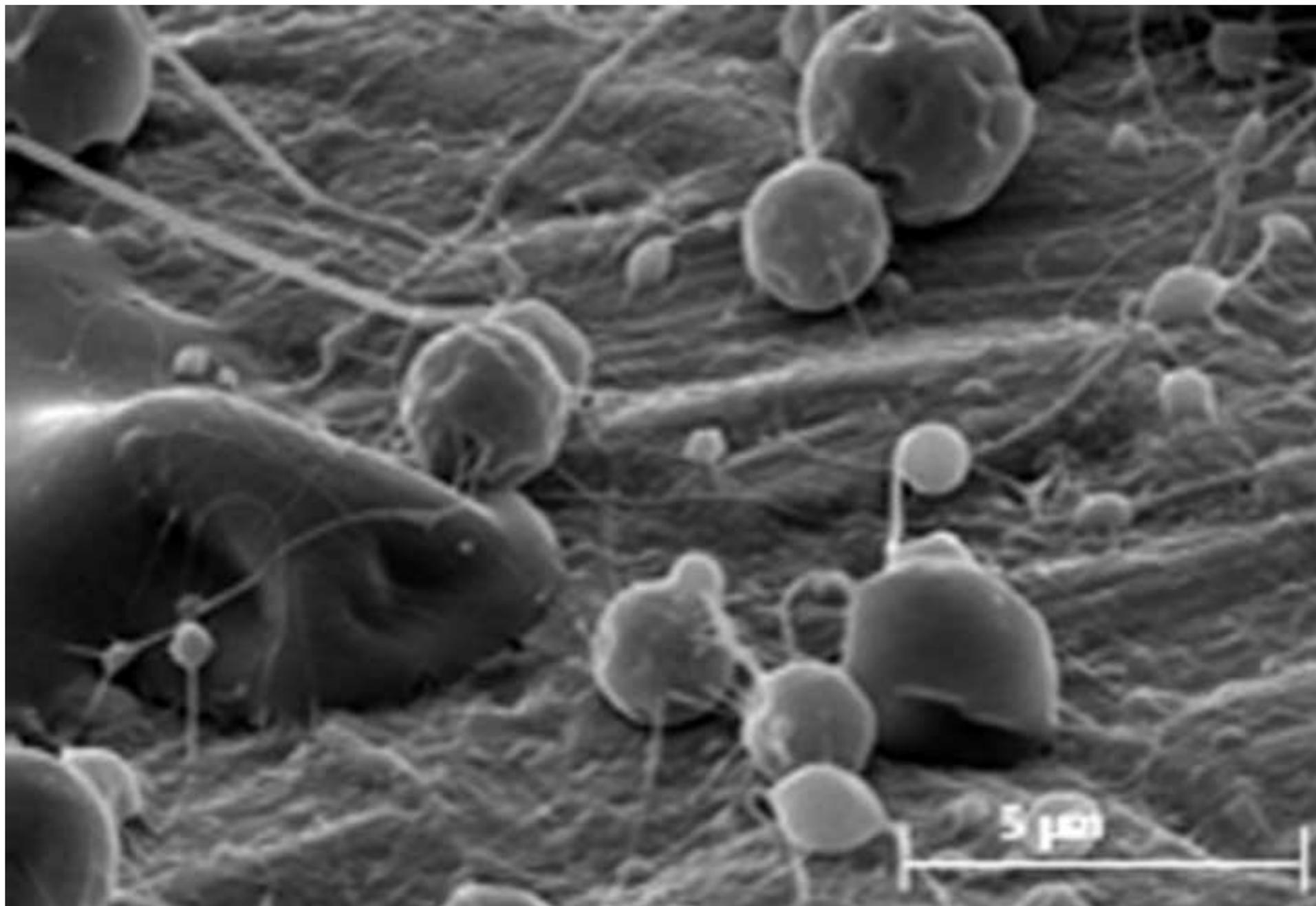


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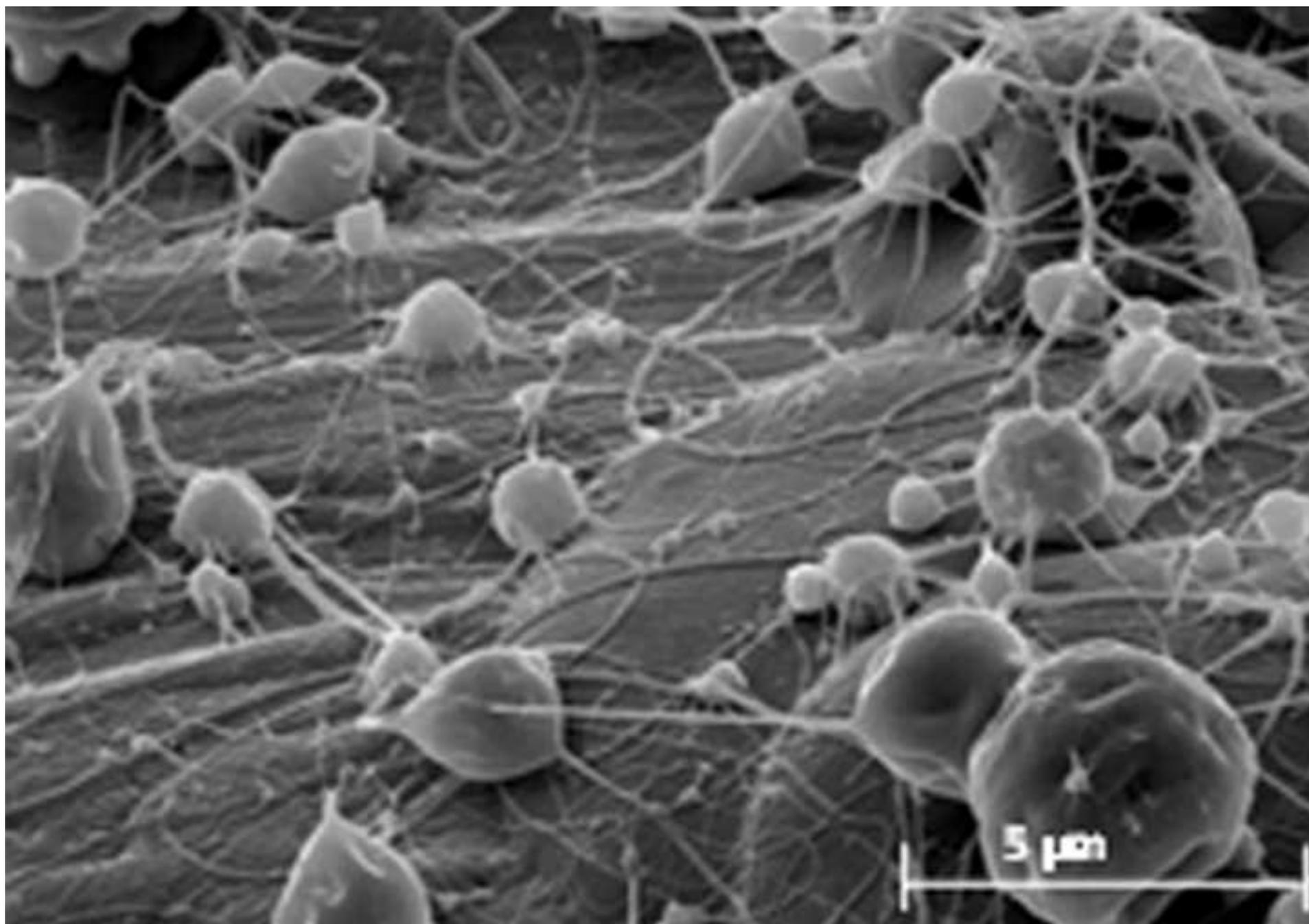


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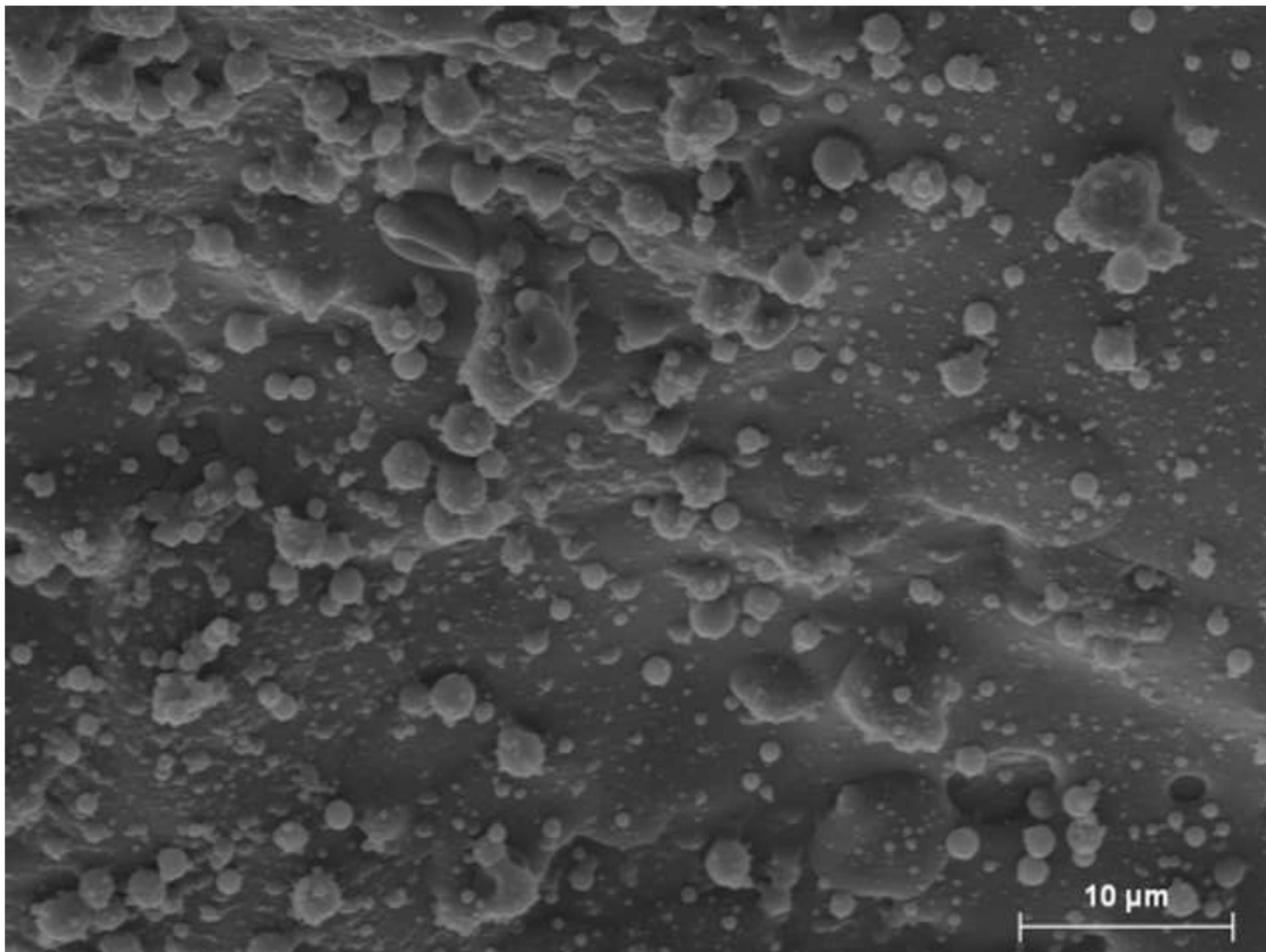


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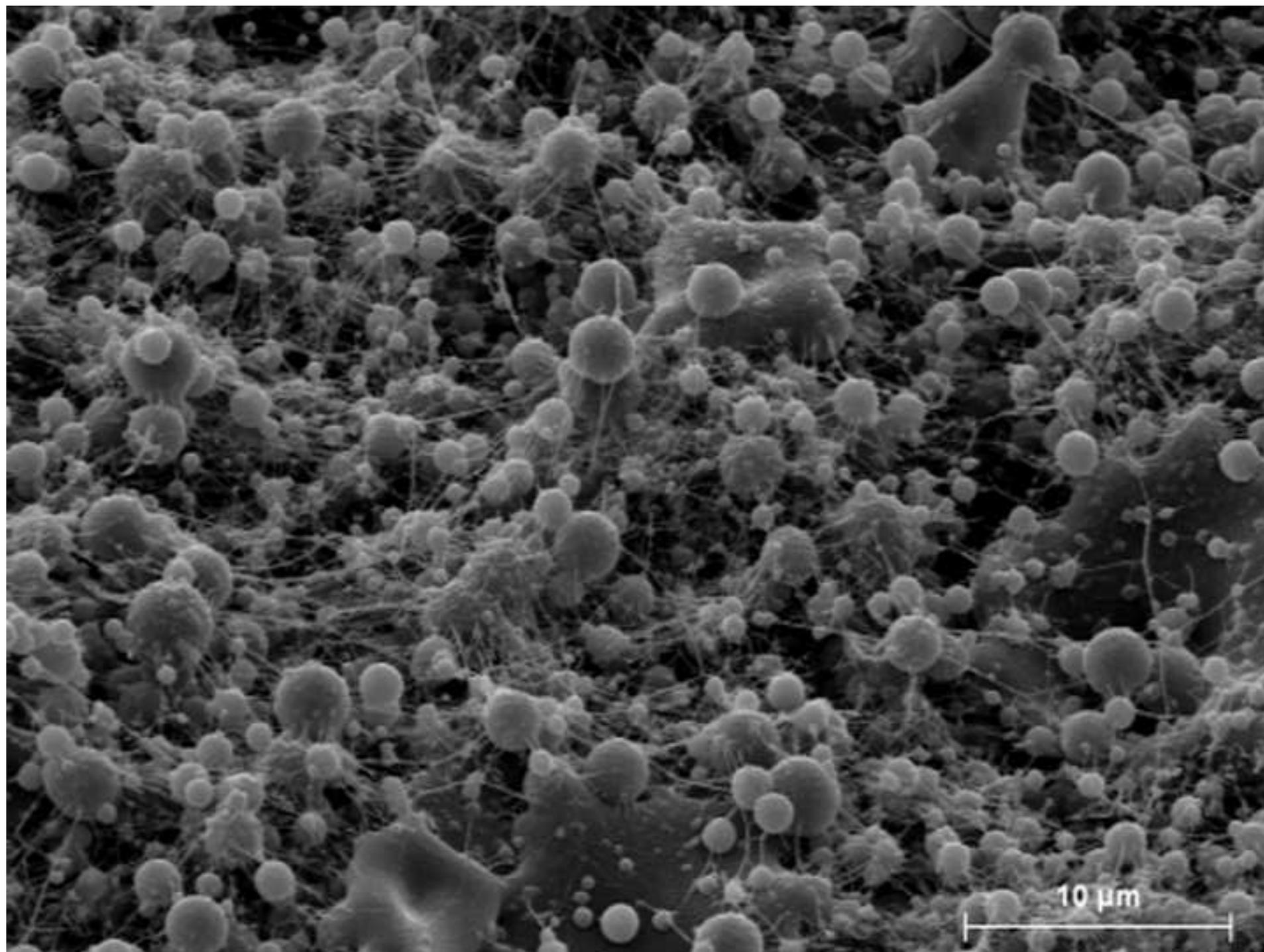


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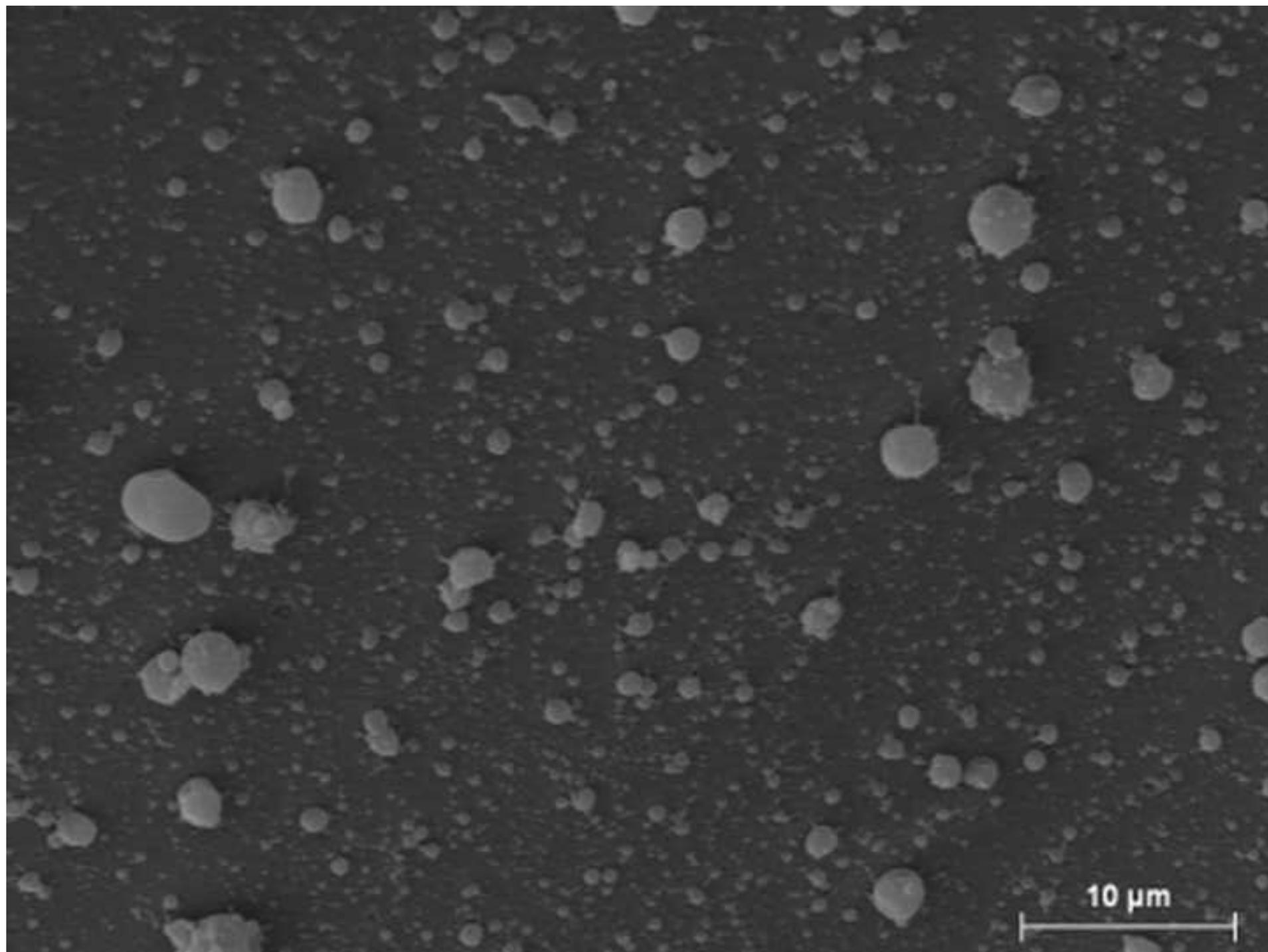


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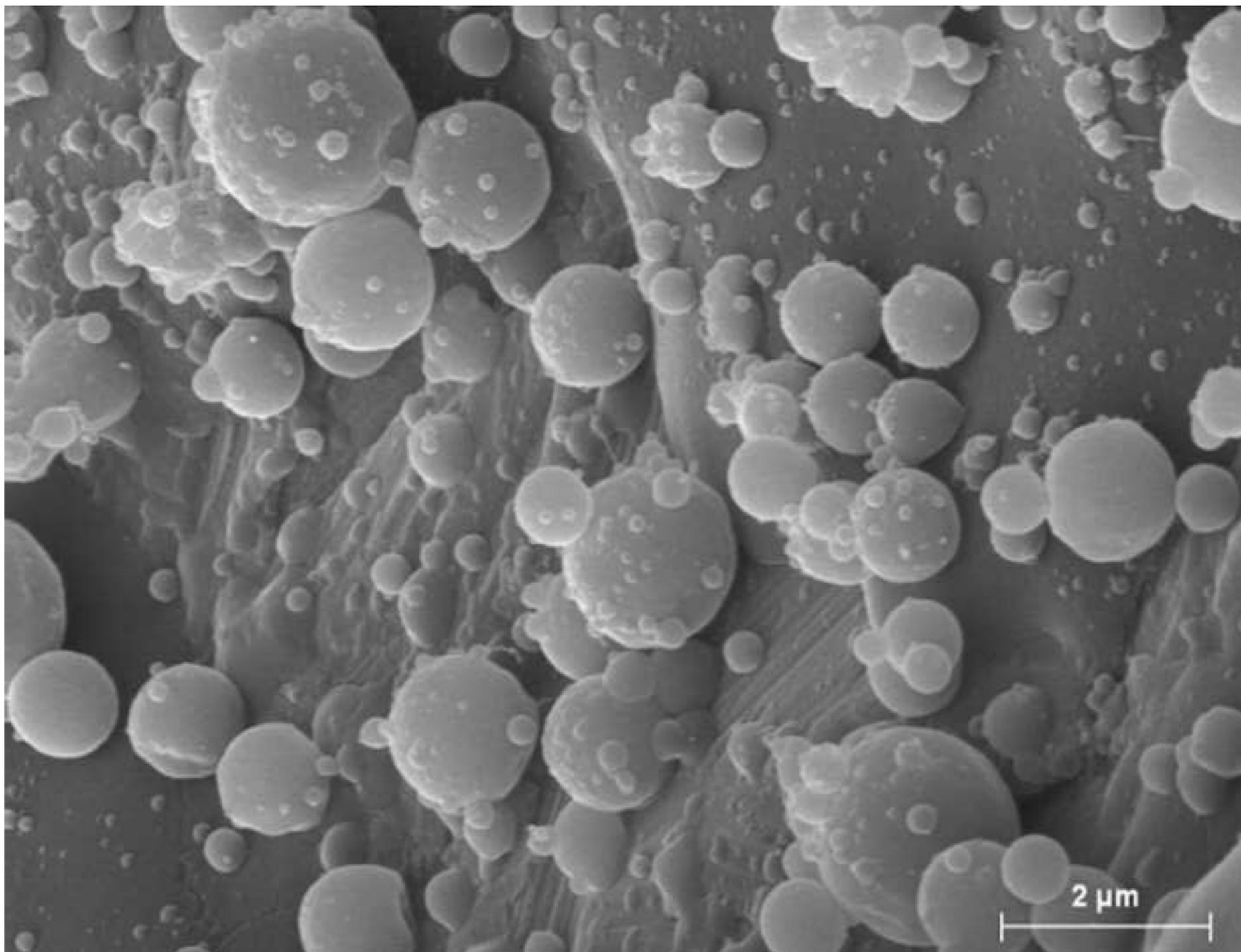


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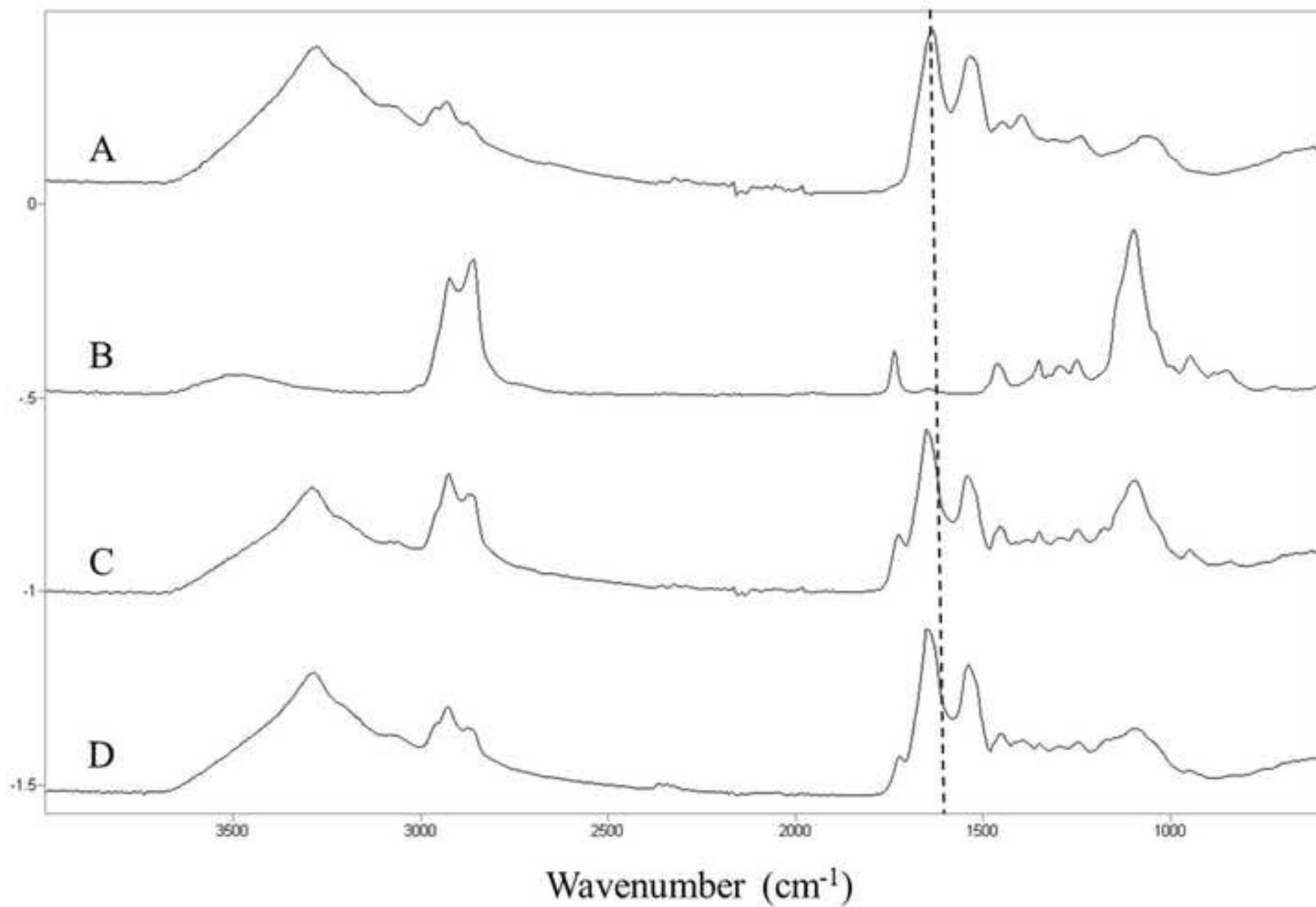


Table 1. Effect of pH and temperature on the amaranth protein isolate solution properties and electrospun structures obtained thereof.

Solvent	%API	Conductivity (μ S)	Surface Tension (mN/m)	Viscosity (cP)	Morphology	Diameter (nm)
Non-heated acetic acid solution (pH 2)	5	1575	33.7 ± 0.4^a	47.9 ± 6.4^a	-	-
Heated acetic acid solution (pH 2)	5	1706	29.9 ± 0.2^b	56.5 ± 8.9^a	Nanoparticles	569.3 ± 182.7^a
Non-heated NaOH 0.01M solution (pH 12)	15	4020	38.9 ± 0.5^c	8.5 ± 5.3^b	Nanoparticles	267.8 ± 82.7^b
Heated NaOH 0.01M solution (pH 12)	15	3610	39.8 ± 0.6^c	21.3 ± 3.5^b	Nanoparticles	169.3 ± 109.9^c

a-c different superscripts within the same column indicate significant differences among samples ($p < 0.05$).

Table 2. Effect of type of solvent and protein concentration on the API solution properties and electrospun structures obtained thereof.

Solvent	% API	Conductivity (μS)	Surface Tension (mN/m)	Viscosity (cP)	Morphology	Diameter (nm)
HFIP	5%	47	18.3 ± 0.2^a	47.2 ± 1.1^a	Beaded fibers	1961.5 ± 996.7^a
	8%	65	18.7 ± 0.3^{ab}	90.5 ± 1.6^b	Fibers	389.3 ± 114.5^b
	10%	101	19.3 ± 0.5^b	166.1 ± 3.8^c	Fibers	-
Formic acid	8%	5950	29.9 ± 0.2^c	92.5 ± 9.4^b	Beads	1375.8 ± 543.5^c
	10%	6820	29.3 ± 0.4^c	110.3 ± 5.8^d	Beads-fibers	1387.6 ± 357.1^c
	20%	8350	29.4 ± 0.2^c	136.6 ± 4.5^e	-	-

The a-e different superscripts within the same column indicate significant differences among samples ($p < 0.05$).

Table 3

Table 3. Effect of surfactant and mercaptoethanol (2-ME) addition on the API-containing formic acid solution properties and electrospun structures obtained thereof.

Solvent + additive	%API	Conductivity (μS)	Surface Tension (mN/m)	Viscosity (cP)	Morphology	Diameter (nm)
Formic acid + 10% Tween80	10	5540	31.3 ± 0.1^a	118.9 ± 2.3^a	Beads	219.7 ± 86.2^a
Formic acid + 20% Tween80	10	5440	30.2 ± 0.4^b	119.5 ± 8.8^a	Beads	369.3 ± 159.9^b
Formic acid + 20% 2-ME	10	5550	30.1 ± 0.1^b	108.2 ± 4.9^a	Beads	-
Formic acid + 20% 2-ME + 15% Tween80	10	5310	30.6 ± 0.2^b	120.9 ± 3.4^a	Beads	738.5 ± 419.1^c

a-c different superscripts within the same column indicate significant differences among samples ($p < 0.05$).

HIGHLIGHTS

- Amaranth protein isolate (API) ultrathin structures were developed by electrospinning
- The effects of pH, solvent and surfactants on morphology and molecular order were studied
- Morphology of electrospun structures was mainly affected by the solution properties
- Surfactant addition improved spinnability and morphology of API ultrathin structures

