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1 **Prebiotic membrane structures mimic the morphology of purported early traces of life on Earth**

2

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14

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16

17 **Key words:** origin of life, micropalaeontology, lipid vesicles, microfossils, biomorphs, biosignatures,
18 prebiotic chemistry, geobiology, protocells, protobiosignatures

19

20 **Abstract**

21 Elucidating the most probable compositions of the first cell membranes prior to the origin of life,
22 within a laboratory setting, requires experiments with organic molecules and chemical conditions
23 representative of those present on the early Earth. As such, the membrane forming molecules used
24 in these experiments are described as 'prebiotically plausible', i.e., they could have formed through
25 abiotic reactions and be available for membrane formation prior to the emergence of biology.
26 Similarly, the chemical properties of solutions in which these membranes are formed (e.g., pH,
27 temperature, ionic strength) must represent the early Earth environmental conditions under
28 investigation. Here, using a combined confocal and transmission electron microscopy approach, we
29 show that prebiotically plausible organic molecules, in solutions representative of Hadean submarine
30 alkaline hydrothermal vents, form aggregated structures with substantial morphological diversity. The
31 structures hold the potential for use as traces of prebiotic processes in the ancient rock record. In
32 addition, many of the structures are morphologically similar to those which are presented as early
33 microfossils, thus highlighting the limitations of morphological interpretation in these types of studies.

34 Detailed analyses of abiotic organic structures are essential for our understanding of the earliest living
35 organisms on Earth, as well as for our interpretation of any potential biosignatures recovered in the
36 future from extra-terrestrial bodies.

37

38 **1. Introduction**

39 There are many theories that exist as to how life on Earth arose. Whether in hot springs on land¹ or
40 hydrothermal vents on the ocean floor^{2,3}, all these ideas require an energy source within a geological
41 setting that can fuel chemical reactions. Over time these reactions increased in complexity from
42 geochemistry to organic chemistry, eventually leading to biochemistry with the emergence of life. The
43 gradients within these settings could potentially drive chemical reactions producing organic molecules
44 from inorganic reactants (e.g.,⁴⁻⁷). Organic compounds then reacted together forming more complex
45 molecules through increasingly advanced pathways, eventually becoming something like a metabolic
46 pathway: a protometabolism⁸. Without boundaries, this protometabolism may not have been long
47 lived. Products could have quickly become diluted, and reactions could have dissipated giving way to
48 alternate reactions. Like the cell membranes found in all living organisms, some form of
49 compartmentalisation would probably have been required. The cell membranes of all extant living
50 organisms are composed of glycerol phosphate phospholipids⁹. However, these phospholipid
51 membranes are possibly too complex to have formed at the earliest stages of the emergence of life
52 on Earth. Instead, early compartments could have been supplied in the form of vesicles, membrane
53 structures composed of single chain amphiphiles (SCAs) such as fatty acids¹⁰, giving rise to the first
54 protocells.

55 It is likely that fatty acids, alcohols, and many other SCAs would have existed in almost any
56 origin of life scenario, and the precursors of membrane-forming molecules may have been synthesised
57 abiotically on the early Earth¹¹. The list of prebiotically plausible organics is ever-growing due to results
58 from both laboratory experiments and analysis of real samples¹². Prebiotic synthesis experiments
59 have achieved the formation of carboxylic acids, amino acids, sugars, and nucleotides^{4,5,13-23}. For
60 example, Fischer-Tropsch-Type (FTT) syntheses under hydrothermal conditions produce numerous
61 fatty acids, alcohols, and alkanes containing 6 to 34 carbon atoms¹³, all of which are suitable
62 membrane-forming components. Carboxylic acids and amino acids of abiotic origin have been
63 detected in rock samples from Earth, while numerous organic molecules including both aliphatic and
64 polyaromatic hydrocarbons (PAHs), hydroxy acids, nucleobases, and amino acids have been detected
65 in meteorites²⁴. Carbonaceous meteorites contain fatty acids, including aliphatic straight-chain and
66 branched-chain monocarboxylic, with up to 12 carbon atoms, and dicarboxylic acids^{25,26}. The straight-

67 chain monocarboxylic acids are dominant, followed by the branched-chain monocarboxylic acids, and
68 finally the dicarboxylic acids. Meteoritic monocarboxylic acids are enriched in deuterium and ^{13}C ,
69 which is consistent with an extra-terrestrial origin. It has been suggested that gas phase reactions in
70 the interstellar medium, involving radicals and ions, synthesised meteoritic monocarboxylic acids
71 before being accreted in the parent body of meteorites for further processing^{27,28}. Carboxylic and
72 dicarboxylic acids can also be formed in the meteorite parent body by two ways: 1) via hydrolysis of
73 carboxamides²⁹; or 2) by the oxidation of hydrocarbons from the macromolecular insoluble organic
74 matter (IOM), or the oxidation of free hydrocarbons by oxidised fluids or minerals³⁰. Vesicles have in
75 fact been formed directly from organics contained within meteorite samples^{31,32}. The meteorite flux
76 to the Earth was much higher during the Hadean than it is today and may have spiked between 4.1
77 and 3.8 Ga^{33,34}. The vast amount of material delivered to the early Earth during this time represents
78 a significant source of organic molecules. Coupled to organics formed *in situ* on the early Earth, it is
79 probable that a wide range of compounds would have been available for membrane formation.

80 Multiple different early Earth environments have been proposed as possible settings for the
81 emergence of the first living organisms. Each of these scenarios brings with it a unique set of
82 conditions that would potentially affect the production and survival of organic molecules, the
83 formation of membranes and the diversity of possible prebiotic chemical reactions. Deep sea locations
84 with hydrothermal activity could provide a supply of organics through FTT syntheses, with high
85 temperature acidic fluids in ‘black smokers’ and lower temperature alkaline fluids in ‘white smokers’
86 providing very different pathways for eventual chemical reactions. Some of these sites may have been
87 too deep for meteoritic organic delivery, while being protected from their impacts. Conversely,
88 terrestrial hot springs are exposed to impacts, while simultaneously being receptive to extra-
89 terrestrial organic delivery. Depending on their geology, these surface sites would have unique pH,
90 temperature, and ionic species in their fluids, each combination of which could be amenable to
91 different modes of prebiotic chemistry. These are just two examples of myriad possibilities. It is clear
92 that the permutations of environmental conditions for potential origin of life locations on the early
93 Earth are vast.

94 Despite this, the majority of work on membrane formation to date has focused on the analysis
95 of vesicles formed from single molecules or simple mixtures containing at most two to three SCAs.
96 The resulting vesicles struggle to survive under environmental stresses such as pH, temperature, and
97 ionic strength fluctuations^{35–38}. Sensitivity to salinity, in particular, has cast doubt on any oceanic
98 environment as a possible origin of life location. Recent work, however, has found that SCAs with
99 novel headgroup moieties can withstand some more challenging conditions including high ionic
100 strength and extremes of pH, particularly at acidic levels³⁹. It has also been shown that combining

101 simple SCAs in relatively complex mixtures, arguably more relevant to a prebiotic scenario, produces
102 vesicles with significant resilience to multiple environmental stresses, including salinity and high pH⁴⁰.
103 Considering the wide range of available SCAs and the vast amount of research that remains to be done
104 on vesicle formation capabilities, it is clearly unreasonable to exclude any potential origin of life
105 environment based on current knowledge. In fact, it now appears likely that vesicles would have
106 formed in almost any prebiotic scenario.

107 Vesicles display a diverse range of morphologies, both on an individual level and as aggregates
108 or clusters of multiple vesicles^{40,41}. These morphologies seem to be affected by environmental
109 conditions. However, this has not been systematically investigated yet. In fact, the primary objective
110 of origin of life membrane formation studies – testing the ability of membrane formation – has meant
111 that these structures have been overlooked, often regarded as failed experiments. Many of these
112 assemblages are reminiscent in their morphology of living organisms and ancient microfossils. Similar
113 forms created entirely from self-assembled nanocrystalline materials have been described
114 previously^{42–45}. It has been suggested that these inorganic ‘biomorphs’ may be observed within the
115 rock record and could be misinterpreted as microfossils of ancient living organisms. The same could
116 be true for organic assemblages of vesicles, and perhaps for biomorphs formed through combinations
117 of both organic and inorganic compounds.

118 Here we show that combinations of prebiotically plausible organic molecules, in solutions
119 chemically representative of a potential early Earth environment, form a variety of complex aggregate
120 structures. Confocal and electron microscopy reveal the morphological diversity of these structures
121 and the similarities they hold with purported microfossils of the earliest living microorganisms on
122 Earth. We highlight the potential for these abiotic structures as diagnostic signatures in their own
123 right, while also highlighting their tendency to mimic microfossils in the rock record. Population
124 morphometry results from organic biomorphs and microbial populations show strong similarities in
125 2D structure. However, differences in population size distribution suggest that this characteristic may
126 be useful in distinguishing between abiotic and biological microstructures of this type. Caution is
127 required when investigating signatures of this nature and significantly more work is required to
128 understand these abiotic structures sufficiently, to ensure that they do not hinder our interpretation
129 of biosignatures both on Earth, and potentially elsewhere in our Solar System in the future.

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134

135 **2. Methodology**

136

137 **2.1 Materials**

138 All reagents used in this study were of analytical grade ($\geq 97\%$) and were procured from either Sigma
139 Aldrich (Merck, UK) or Acros Organics (UK).

140

141 **2.2 Preparation of vesicle solutions**

142 The vesicle solutions were prepared employing a modified version of the technique described by
143 Monnard and Deamer (2003)⁴⁶. Glass vials were employed for all solution preparations in a dry heating
144 block maintained at a temperature of 70 °C. This temperature corresponds to the conditions expected
145 at Hadean alkaline hydrothermal vents (50 – 100 °C)⁴⁷. The molecules used were fatty acids and 1-
146 alkanols (C₁₀ to C₁₅, odd and even), and the isoprenoid molecules geranic acid and geraniol. The lipids
147 were heated and introduced to the solutions in their liquid state. Acid molecules were first added to
148 deionized (DI) H₂O to provide the desired final concentration and subjected to vortexing.
149 Subsequently, 1 M NaOH was added until the solution turned transparent, indicating complete
150 deprotonation of the acid. The alcohols were then added, and the solution was vortexed. The pH was
151 adjusted by employing 1 M HCl or 1 M NaOH to achieve the desired final value. The solution was then
152 brought to the desired final volume by adding DI H₂O. Immediate analysis of solutions was conducted
153 after their preparation.

154

155 For the preparation of vesicles in salt solutions, the H₂O was substituted with the desired salt
156 concentration, and the acid and base solutions were substituted with the salt dissolved in 1 M HCl and
157 1 M NaOH, respectively, to ensure consistent salt concentrations throughout. Anoxic vesicle solutions
158 (H₂O, FeCl₂, Na₂S, FeS, Fe particles) were prepared following the aforementioned method in an
159 anaerobic hood with a 5% H₂ in N₂ atmosphere. O₂ was eliminated by reacting with H₂ to form H₂O
160 vapour on a Pd catalyst. O₂ levels were monitored and maintained at 0 ppm throughout these
161 procedures. FeS solutions were prepared using a 1:1 ratio of FeCl₂ and Na₂S. Fe particles were added
162 to the relevant solutions after vesicle formation in H₂O. All anoxic solutions were sealed with lids and
163 wrapped in parafilm. The seal was broken immediately before imaging.

164

165 **2.3 Confocal microscopy**

166 Confocal microscopy was conducted using a Zeiss LSM-T-PMT 880 instrument coupled to an Airyscan
167 detector. Membranes were visualised using the hydrophobic dye Rhodamine 6G. 0.5 µL of a 100 µM
168 dye solution was added to a heated (70 °C) microscope slide. The vesicle solution was vortexed, and

169 a 5 μ L aliquot was added to the slide, mixed with the dye. The aliquot was covered with a #1.5, 16 mm
170 diameter coverslip and positioned on the microscope stage. The dye was excited using an Ar laser
171 operating at 514 nm and observed through a 63x oil objective with a 488 nm filter. Images were
172 captured using Zeiss Zen microscopy software, and final processing was conducted using the FIJI (Fiji
173 is just ImageJ) software package.

174

175 **2.4 Negative Staining – Transmission Electron Microscopy (NS-TEM)**

176 NS-TEM was performed using a JEOL 1010 TEM (JEOL, Japan). Samples were applied to a Cu 100 mesh
177 grid and allowed to incubate for 30 seconds. Excess sample was then removed by blotting with filter
178 paper, and a portion of aqueous uranyl acetate (1.5%) was added to the grid. After standing for 30
179 seconds, the excess liquid was blotted. Grids were immediately analysed under vacuum. Image
180 processing was carried out using the FIJI software package.

181

182 **2.5 Cryo-electron microscopy (Cryo-EM)**

183 Samples were prepared using a Vitrobot Mark IV (Thermo Fisher). An aliquot was applied to a glow-
184 discharged Lacey Carbon (400 mesh Cu) grid (Agar Scientific) for 30 s, blotted for 8.5 or 11 s at 4.5 $^{\circ}$ C
185 and 95% humidity, and then rapidly plunged into liquid ethane. Imaging was performed on a T10
186 microscope (FEI) at 100 kV. Images were collected at a magnification range of 7000-34000x.

187

188 **2.5 Population morphometry**

189 Population morphometry analysis^{43,48,49} was performed on several images of the experiments,
190 following the protocols described in Rouillard et al. (2020)⁴⁹. All image treatment was carried out using
191 FIJI version 1.53t. Some improvements in image segmentation were implemented, relative to
192 Rouillard et al. (2020)⁴⁹, particularly making use of machine learning trainable segmentation and
193 threshold binarization. The individual steps of morphometric analysis are described below:

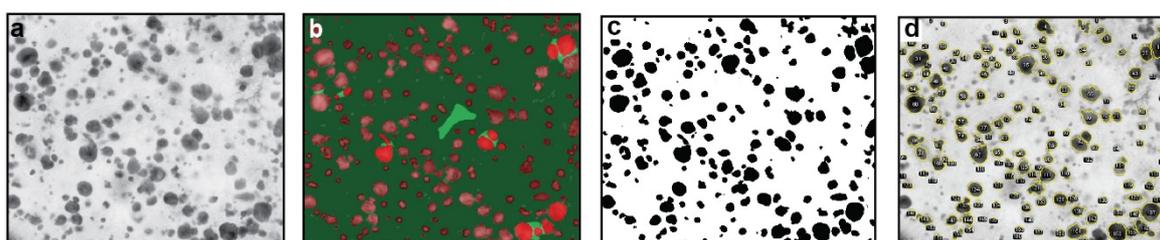
194

195 *2.5.1 Image segmentation*

196 Using FIJI, each image was cropped to exclude scale bars and other annotated features. For some
197 images with uneven background shading, a subtraction of background noise was performed using the
198 Subtract Background process (using a rolling ball radius of approximately the size of the particles).
199 Further optimisation before segmentation depended on the individual image. This included image
200 inversion (Fig. S1b), an additional Gaussian blur to make cells stand out relative to artifacts (Fig. S2b),
201 or edge-detection (using the Canny Edge plugin) followed by hole-filling (Fig. S3c).

202 Subsequently the Trainable Weka Segmentation plugin⁵⁰ was loaded. The classifier training process
203 was initiated using two classes, one for the structures of interest and another for background (Fig. 1;
204 Fig. S1 c, d; Fig. S2 c, d). The classifier was trained to fill holes and to separate touching cells. In some
205 images it was more effective to classify artifacts that obscured the cells (Fig. 3; Fig. S3c-f). In that case
206 an image was created that could be subtracted from the pre-Weka image, resulting in a cleaned-up
207 version showing only the particles of interest. For all Weka-segmented images a binarized segmented
208 image was then created (Fig. S1e, S2e, S3g). Individual particles could then be counted using the
209 Analyze Particles option, selecting area and shape descriptors. The major steps of this protocol are
210 shown in Figure 1.

211



212

213 *Figure 1. Major steps in image segmentation and particle counting protocol. Experimental vesicles*
214 *(dried). a) Original, cropped image, b) Weka trainable segmentation, separating individual cells from*
215 *background, c) thresholding and binarization, d) Particle counting, size and shape description. For more*
216 *details on segmentation, see Figure S1.*

217

218 2.5.2 Particle characterization

219 Following Rouillard et al. (2020)⁴⁸, for each segmented image the entire population of particles was
220 counted (using a 5-pixel size threshold to avoid small dots), and for each particle the area (A) (in square
221 μm), radius (R) (assuming a perfect circle, in μm) circularity (C) and solidity (S) were determined.
222 Circularity is defined as $C = 4\pi A/P^2$, where P (in pixels) is the circumference of the particle. Solidity is
223 defined as $S = A/\alpha$ where α is the area (in square pixels) within the convex hull of the particle. This
224 convex hull consists of the surface bound by straight lines that join the outermost points of the
225 particle.

226

227 Results

228 A selection of prebiotically plausible and biochemically important single chain amphiphilic organic
229 molecules were used to investigate the formation of abiotic structures (Table 1). Different
230 combinations of organics, pH and additional ionic species were used for each solution prepared.
231 Molecules were first dissolved in alkaline (pH ca. 11) aqueous NaOH solutions. This pH is sufficiently

232 above the pKa of the individual molecules to provide solutions so that the organics exist as monomers
233 or micelles. The solutions were titrated with 1 M HCl to encourage bilayer formation leading to the
234 development of individual vesicles and more complex structural assemblages. Confocal micrographs
235 of 'typical' solutions present with multiple circular and elliptical vesicles (Fig. 2a-c). Some vesicles can
236 become elongated and others encapsulated within larger vesicles (Fig. 2b and c). Vesicles are generally
237 floating in solution unobstructed during analysis and display some flow caused by capillary action on
238 the glass slide and erratic movement likely due to Brownian motion (SI video). TEM is performed under
239 vacuum and as such the resulting micrographs contain collapsed or 'doughnut' shaped vesicles (Fig.
240 2d-f). These would have been spherical in shape prior to being exposed to the vacuum pressure.

241 Table 1. Full details for each figure panel. SCA – single-chain amphiphile; FA – fatty acid; OH – alcohol; GOH – geraniol.

Figure	Microscopy	Scale bar	SCAs	pH	Formation temperature (°C)	Ionic species	Additional components	Morphologies	Size range
2 (a)	confocal	10 μm	C12 + C14 FAs, C10 OH	7.5	70	NA	Tris buffer	circular, elliptical, encapsulated	<10 μm
2 (b)	confocal	10 μm	C10 FA and OH	7.8	70	NA	NA	circular, elliptical, encapsulated, elongated, chains	<10 μm
2 (c)	confocal	10 μm	C10 FA, C10 OH	7.8	70	NA	NA	circular, elliptical, encapsulated, elongated	<10 μm
2 (d)	transmission electron	1 μm	C10 FA, C10 OH	12	70	HCO ³⁻	NA	circular, elongated, chains	<1 μm
2 (e)	transmission electron	1 μm	C10, C11, C12, C13, C14, C15 FAs	7.5	70	NA	NA	collapsed/ "doughnut"	<1 μm
2 (f)	transmission electron	200 nm	C10, C11, C12, C13, C14, C15 FAs and OHs	8.8	70	NA	NA	collapsed/ "doughnut"	ca. 200 nm
3 (a)	confocal	10 μm	C10, C11, C12, C13, C14, C15 FAs and OHs	9.5	70	NA	NA	circular, laminated, curved-rod	ca. <1 to 50 μm

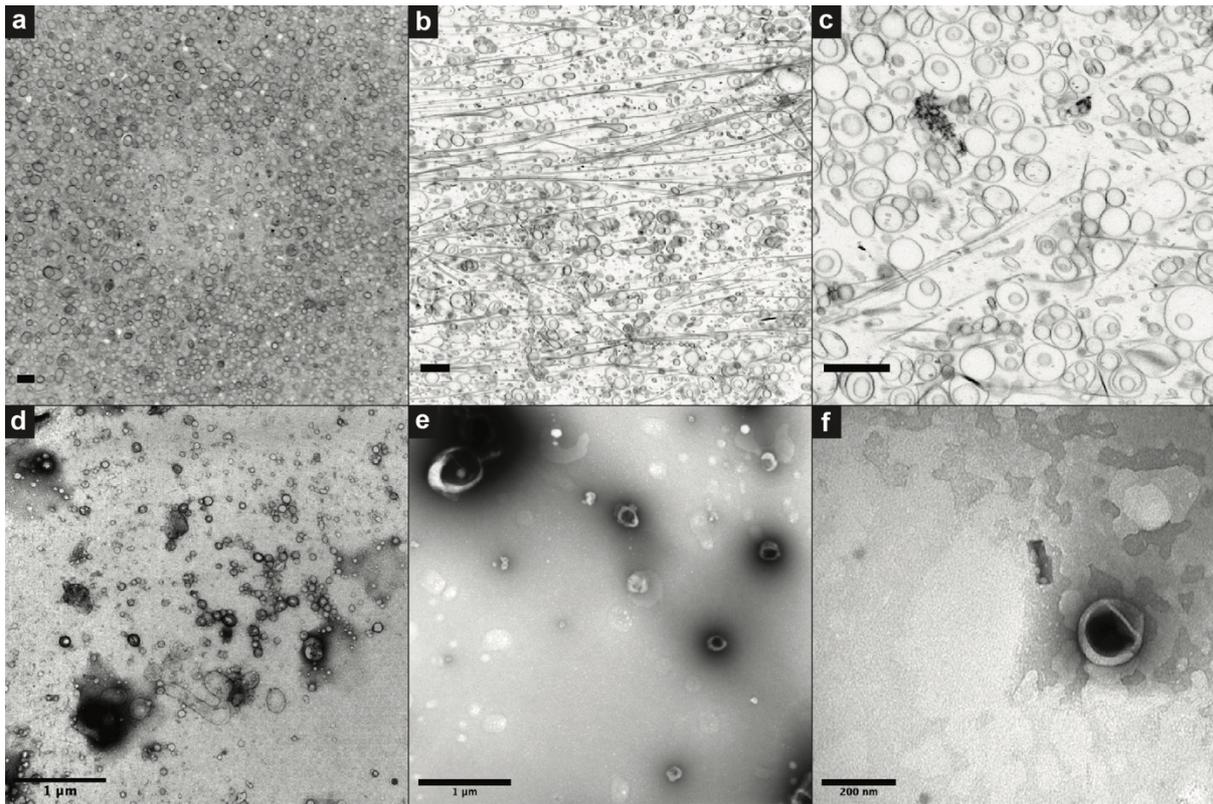
3 (b)	confocal	10 μm	C10, C11, C12, C13, C14, C15 FAs	9.3	70	NA	NA	chains, filaments, clusters	ca. 2 to >50 μm
3 (c)	confocal	10 μm	C10, C11, C12, C13, C14, C15 FAs	8.4	70	NA	NA	chains, filaments, clusters	ca. 2 to >50 μm
3 (d)	confocal	10 μm	C10, C11, C12, C13, C14, C15 FAs and OHs	8	70	NA	NA	clusters, appendages	<10 μm
3 (e)	confocal	10 μm	C10 FA and OH	7.3	70	NA	Tris buffer	circular, elliptical, aligned	<10 μm
3 (f)	confocal	10 μm	C10 FA and GA	13	70	HCO ³⁻	NA	circular, elliptical, filaments, chains	<100 μm
4 (a)	transmission electron	1 μm	C10, C11, C12, C13, C14, C15 FAs	ca. 12	70	NaCl	NA	filaments/chains	>10 μm
4 (b)	transmission electron	200 nm	C10, C11, C12, C13, C14, C15 FAs and OHs	9.1	70	NA	cysteine	filaments, collapsed/ "doughnut"	<1 μm
4 (c)	transmission electron	1 μm	C10, C11, C12, C13, C14, C15 FAs and OHs	12	70	NaCl	NA	circular, symmetrical	<1 μm

4 (d)	transmission electron	10 μm	C10 FA and GOH	12	70	FeCl_3	NA	filaments, clusters, dendritic	<50 μm
4 (e)	transmission electron	1 μm	C10 FA and GOH	12	70	HCO_3^-	NA	circular, collapsed/"doughnut", clusters	<1 μm
4 (f)	transmission electron	1 μm	C10, C11, C12, C13, C14, C15 FAs	11	70	Hydrothermal seawater ⁵¹		circular, collapsed/"doughnut"	<100 nm
7 (a)	petrographic ⁵²	10 μm	NA	NA	NA	NA	suggested microfossils	circular, elliptical, clusters	<5 μm
7 (b)	petrographic ⁵³	10 μm	NA	NA	NA	NA	suggested microfossils	circular, elongated, clusters	<5 μm
7 (c)	petrographic ⁵⁴	10 μm	NA	NA	NA	NA	suggested microfossils	irregular circles	ca. 10 μm
7 (d)	scanning electron ⁵⁵	10 μm	NA	NA	NA	NA	biomorphs	circular, clusters, filaments	<5 μm to >50 μm
7 (e)	confocal	10 μm	C10, C11, C12, C13, C14, C15 FAs and OHs	9.1	70	NA	NA	circular, elongated, clusters	<10 μm

7 (f)	confocal	10 μm	C10 FA and OH	7.4	70	FeCl_3	NA	circular, elliptical, elongated, clusters, encapsulated	<10 μm
7 (g)	confocal	10 μm	C10 FA and GOH	7	70	CaCl_2	NA	circular, irregular circles, filament, encapsulated	<10 μm
7 (h)	transmission electron	10 μm	C10, C11, C12, C13, C14, C15 FAs	ca. 12	70	NaCl	NA	filaments	>50 μm

242

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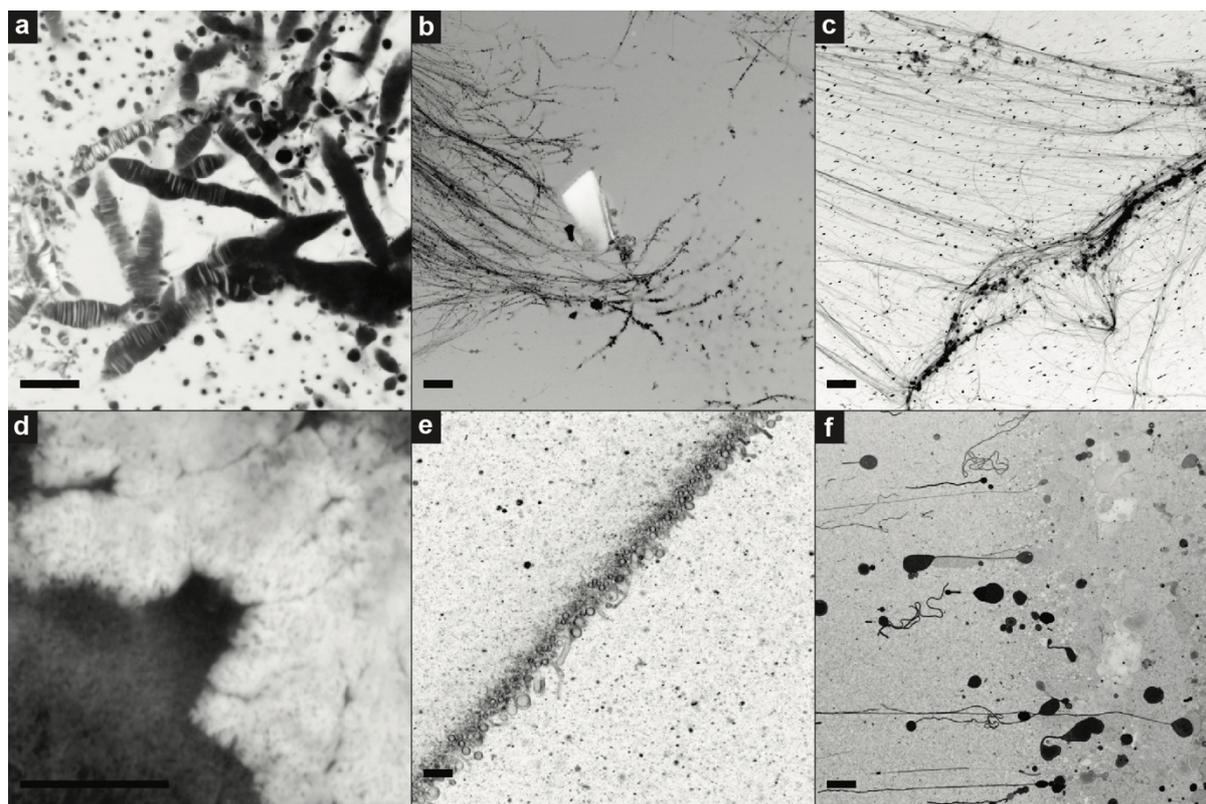
244

245 *Figure 2. Micrographs of images from ‘successful’ vesicle formation experiments. (a-c) Vesicles in*
246 *solution imaged using confocal microscopy, (d-f) transmission electron micrographs of vesicles*
247 *mounted on Cu grids. Scale bars are 10 μm (a-c), 1 μm (d and e) and 200 nm (f).*

248

249 As fluid flows on the glass slide during confocal analysis, some vesicles remain in place
250 adhering to the glass and subsequently drying out. This can leave traces in a variety of shapes including
251 laminated, curved-rods (Fig. 3a), large clusters with appendages (Fig. 3d), and filled circles or ellipses
252 connected to filaments (Fig. 3f). Filaments are a common occurrence in solution (Fig. 3b and c)
253 although their formation mechanism is unclear. Individual vesicles can also settle in formation while
254 still in solution (e.g. linear formation in fig. 3e). This is likely due to a physical deformation in the glass
255 slide which is linear in shape or the presence of a linear contaminant object to which the vesicles are
256 attracted, potentially by surface charge interactions. Further work is necessary to elucidate the exact
257 mechanisms behind these formations.

258

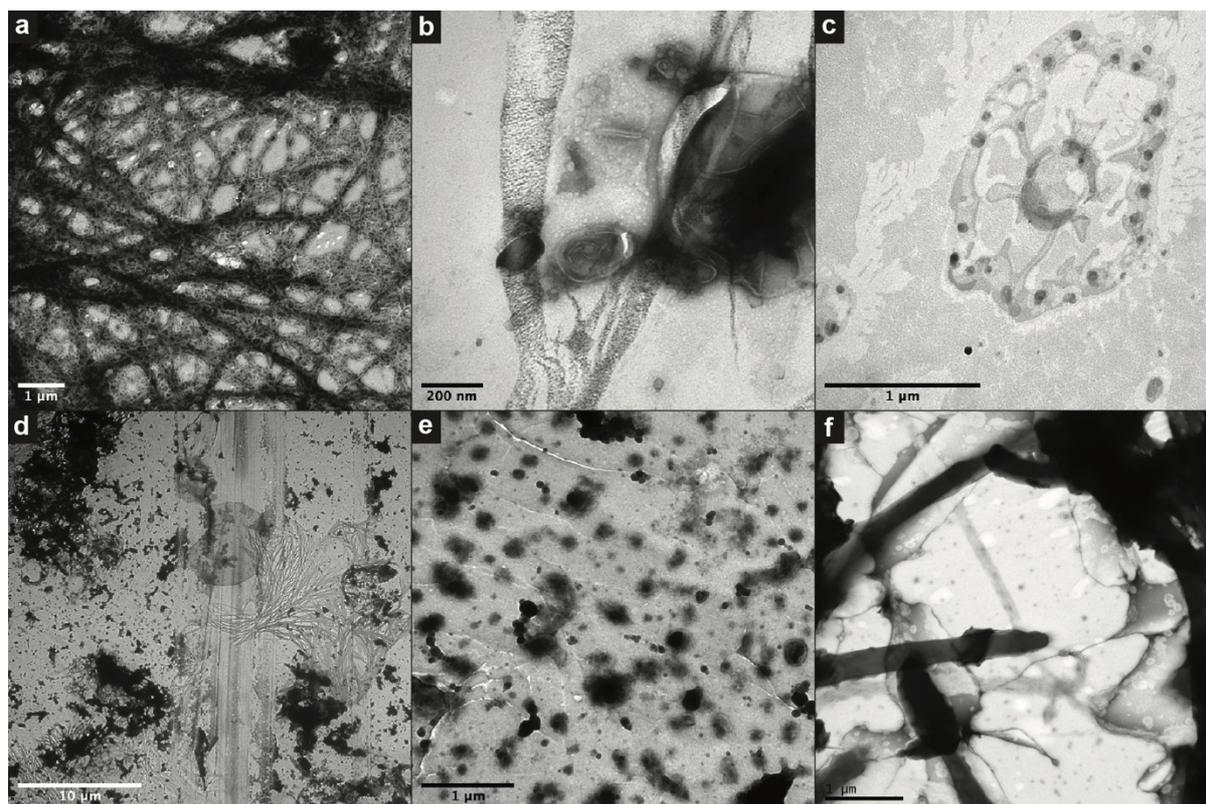


259

260 *Figure 3. Confocal micrographs of aggregates formed from solutions of organic molecules used in*
261 *vesicle formation experiments. All scale bars are 10 μ m.*

262

263 The diversity of assemblage morphologies increases when analysed by TEM (Fig. 4). The
264 vacuum clearly plays an important role here as in the drying during confocal analysis which also
265 produced unique structures. Collapsed or 'doughnut' structures remain common but are often
266 accompanied by filaments, chains, dendritic, and symmetrical patterns. The cause of these patterns is
267 unclear although the presence of inorganic components in the solutions tends to increase the
268 likelihood of complex morphologies. Some of the filamentous structures are comprised of individual
269 vesicles and have been observed previously during confocal microscopy and subsequent TEM of the
270 same solutions⁴⁰. It was suggested that these filaments are the result of the organic vesicles being
271 essentially salted-out of solution by the presence of inorganic salts such as NaCl.



272
273 *Figure 4. Transmission electron micrographs of aggregates formed from solutions of organic molecules*
274 *used in vesicle formation experiments. Scale bars are 1 μm (a, c, e and f), 200 nm (b) and 10 μm (d).*
275

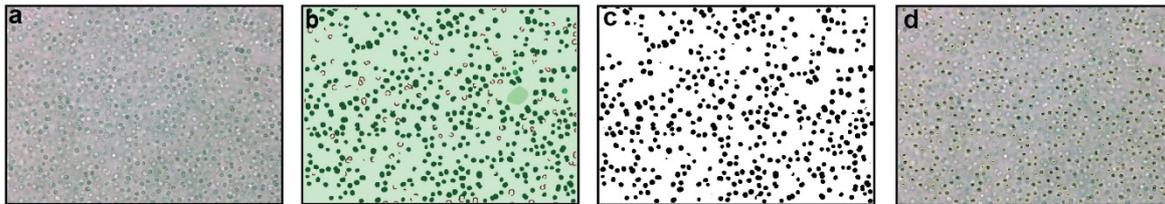
276 The population morphometry results for experimental vesicles were compared with a
277 population of coccoidal cyanobacteria (Fig. 3a in Ref⁴⁸,). For this latter image the exact same (updated)
278 segmentation protocol was used (Fig. 5). The results are shown in Table 2, Fig. 6 and Fig. S4. Both
279 circularity (C) and solidity (S) values for biomorph images (C = 0.83 – 0.84, S = 0.90 – 0.95) are similar
280 to those of the cyanobacteria (C = 0.88, S = 0.96). The primary difference between biomorphs and
281 microorganism in these analyses lies in the size measurement, where the cyanobacteria have a narrow
282 size distribution (Fig. 6c, Fig. S4, radius mean/Std = 5.37) while the biomorphs have a radius mean/Std
283 = 2.33 and radius mean/Std = 1.55 for dried and cryo experiments, respectively (Fig. 6b, c; Fig. S4).
284 Overall, these data show that these organic biomorphs have a less uniform size distribution than
285 typical single-strain populations of microbial cells, but that their shape may be indistinguishable. Given
286 that a single parameter is not a reliable indicator, further experimentation is needed to expand our
287 understanding of population morphometry in this area. Using a broader range of biomorphs, microbial
288 populations, and microfossils will allow us to determine if there are statistically significant differences
289 that will enable this approach to be used to determine biogenicity in the future.

290
291

292 *Table 2. Particle characterisation parameters for Synechocystis sp. and biomorphs*

Sample	ID	Rad. M	Rad. Std	Rad. M/Std	Circ. M	Circ. Std	Circ. M/Std	Sol. M	Sol. Std	Sol. M/Std
Synechocystis	PCC	1.40	0.26	5.37	0.88	0.08	10.99	0.96	0.03	31.20
Biomorph-dried	DV	2.12	0.91	2.33	0.84	0.13	6.54	0.90	0.04	20.51
Biomorph-cryo	CV	14.40	9.28	1.55	0.83	0.09	9.57	0.95	0.03	30.89

293



294

295 *Figure 5. Image treatment for Synechocystis sp. (PCC6803), following the same protocol as for the*
296 *experimental vesicles, shown in Fig. 1a) Original, cropped image, b) Weka trainable segmentation,*
297 *separating artifacts from cells and background (see Fig. S3 for details), c) Tresholding and binarization,*
298 *d) Particle counting, size and shape description. For more details on segmentation see Figure S3.*

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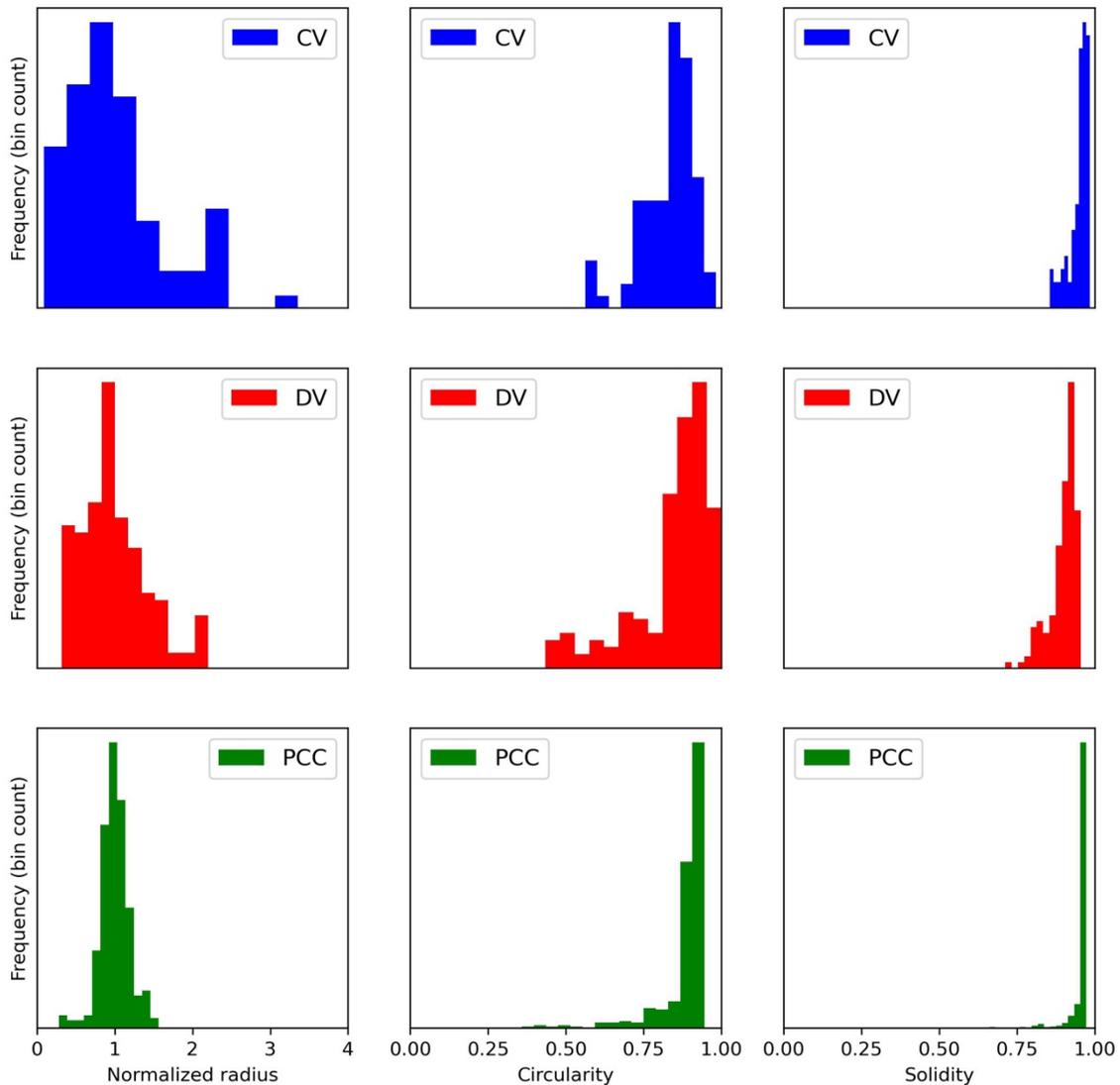
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311 Figure 6. Distributions of normalized size, circularity and solidity of cryogenic vesicles (Fig. S2), dried
312 vesicles (Fig. S1) and microbial cells (*Synechocystis sp.*, PCC6803, Fig. S3).

313

314 Discussion

315 Our results show that abiotic organic molecules, likely present on the early Earth, including fatty acids,
316 1-alkanols, and isoprenoids are capable of forming a diverse range of complex morphologies. Confocal
317 microscopy and TEM analyses provide insights into the possible forms observed using different
318 analytical techniques. The microstructures are formed across a range of environmental conditions
319 with varying pH, temperature, and ionic strength – each of which appear to affect structural
320 morphology. The vast possible combinations of abiotic organic molecules, inorganic materials, and
321 chemical conditions on the early Earth suggest that the formation of these kinds of biomorphs would
322 have been inevitable. Yet our understanding of their formation mechanisms and diagnostic
323 characteristics remains in its infancy, primarily due to a paucity of research in this area.

324

325 *Comparison with microfossils*

326 From the relatively small selection of abiotic mixtures presented here, a substantially diverse range of
327 morphologies was observed some of which are reminiscent of suggested microfossils from Earth's
328 early rock record (Fig. 7). Vesicle experiments were not directed in any way towards the replication of
329 these or any microfossil morphologies. Yet, many microfossil morphologies reported in the Archean
330 and Paleoproterozoic rock record appear to have comparable shapes and sizes. Clusters of
331 carbonaceous spheroids and filaments, interpreted as ancient cell colonies, have been reported in e.g.
332 3.4-3.2 Ga cherts of the Barberton Greenstone Belt (BGB), South Africa^{52,56-58}, 3.5-3.0 Ga cherts of the
333 Pilbara Granitoid-Greenstone Belt, Western Australia^{53,54,59-64}, and the 1.9 Ga Gunflint Formation,
334 Canada^{53,65,66}. Some examples of morphological comparison between microfossils and
335 experimentally-produced vesicles are shown in Figure 7.

336 For instance, spheroidal or spherule-like microfossils in the ca. 3.3-3.4 Ga Kromberg Formation
337 within the BGB (Fig. 7a)⁵², are similar in shape to many abiotic vesicle micrographs including the
338 example presented in Figure 5e which were formed from a mix of twelve fatty acids and 1-alkanols
339 (C10 to C15) at alkaline pH (pH 9.1). Westall et al. (2001)⁵² present several examples of spherules
340 'joined in pairs', a feature which is also observed in vesicle solutions. They argue that their observed
341 structures fulfil several biological criteria including size, morphology, cell division, colonial distribution,
342 and cell wall texture. While we agree with this assessment, the vesicle experiments here also arguably
343 meet these criteria. In another example, clusters of spheroidal microfossils, identified in the ca. 3.4
344 Ga Strelley Pool Formation, Western Australia, and interpreted as remnants of sulphur-metabolising
345 cells³⁴ (Fig. 7c), are similar in shape to the ca. 10 µm diameter circular vesicles formed in the laboratory
346 from decanoic and geranic acid in the presence of CaCl₂ (Fig. 7g).

347 These morphological similarities extend to filamentous shapes and even to mixtures of
348 spheroids and filaments. For example, Wacey et al. (2012)⁵³ reported a selection of spheroidal and
349 filamentous microfossils (suggested Huroniospora and Siphonophycus/Gunflintia respectively) from
350 within a stromatolitic chert of the ca. 1.9 Ga Gunflint Formation, Canada (Fig. 7b). We also observed
351 these co-occurring morphologies in solutions of decanoic acid and 1-decanol containing FeCl₃ at pH
352 7.4 (Fig. 7g), although combined circular and filamentous vesicle morphologies are not restricted to
353 Fe-containing solutions. Considering the younger age of these microfossils a biogenic origin is likely
354 but without more advanced data on the formation and preservation of abiotic microstructures it
355 remains uncertain.

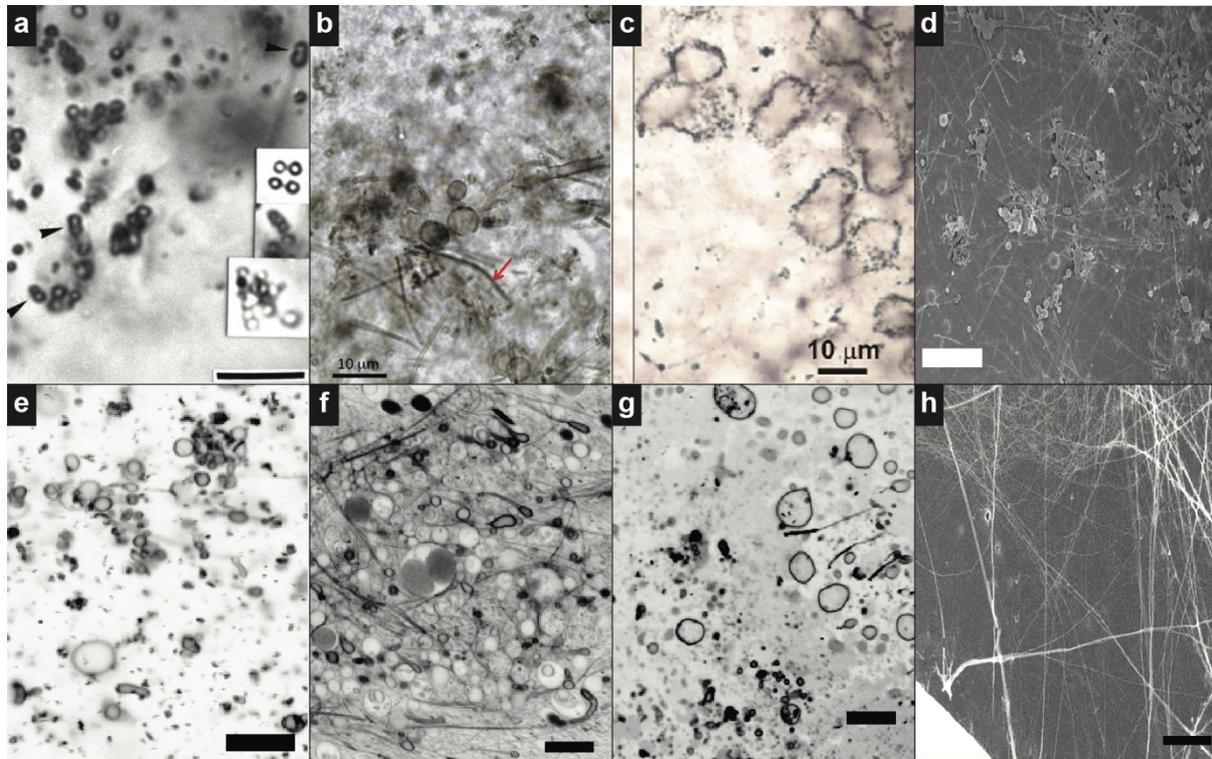
356 It should be noted that, although the vast majority of Archean and Paleoproterozoic
357 microfossil morphologies are spheroidal or filamentous, other morphologies exist such as spindle-like

358 and lenticular shapes^{61,67,68} and hollow tubular organic structures⁶⁹, that do not appear to be
359 mimicked in our current experiments. It may be possible that these morphologies result specifically
360 from the larger molecules which make up prokaryotic cell walls (i.e., polysaccharides and proteins).
361 Indeed, much of the biogenicity criteria employed for microfossil interpretation relates to interpreting
362 cell wall morphology specifically. Further work is needed to elucidate this possibility. That lipid vesicles
363 are chemically analogous to cell membranes rather than cell walls should not however affect their
364 relevance to microfossil interpretation. In chemical terms, following billions of years of diagenesis,
365 organic molecules will largely have been converted to graphite regardless of the complexity of their
366 original form. Some of the biomorph morphologies presented here result from individual vesicles
367 forming individual spherical shapes (e.g., Fig. 2a). Others, such as the filamentous structures, are
368 formed from aggregates of individual vesicles (e.g., Fig. 4a). In addition, it has been shown previously
369 that vesicles can bind to and coat mineral particles, likely due to surface charge interactions, creating
370 an organic cast of the mineral shape⁴¹. Self-assembly of abiotic SCAs may yet yield these additional
371 morphologies, particularly in conjunction with relevant inorganic species. Moreover, detailed physical
372 and chemical analyses of organic biomorphs will allow us to better understand their relevance to
373 microfossil interpretation.

374 It is important to realize that up to this point only the morphological aspects of these
375 abiogenic vesicles have been studied and compared to ancient microfossils. In recent years, however,
376 it has become possible to image and characterize individual microfossils using in-situ analytical
377 techniques such as Raman spectroscopy, confocal laser scanning microscopy (CLSM), Secondary ion
378 mass spectrometry (SIMS, NanoSIMS), transmission electron microscopy and energy-dispersive X-ray
379 spectroscopy (TEM-EDXS), and synchrotron-based scanning transmission X-ray spectroscopy (STXM).
380 Combinations of these techniques make it possible to directly link chemical and isotopic
381 characteristics to the morphology of microfossils. A recent overview of these techniques for the study
382 of microfossils is given in Lepot (2020)⁷⁰. It is thus important to note that some of the examples of
383 microfossils given above have been studied using additional chemical and isotopic analyses. For the
384 spheroidal microfossils identified by Wacey et al. (2011)⁵⁴ in the ca. 3.4 Ga Strelley Pool Formation,
385 Western Australia (Fig. 7c), specific SIMS-based $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ analyses provided evidence for sulphur-
386 based metabolism. For the filamentous and spheroidal microfossils of the 1.9 Ga Gunflint Formation
387 Wacey et al. (2012)⁵³ (Fig. 7b) performed more advanced morphological and elemental analyses
388 utilising focused ion beam (FIB) milling in combination with SEM and TEM to provide micrographs of
389 both nanoscale resolution and in three dimensions. In general, a range of chemical- and isotopic
390 biogenicity criteria have thus been created that can be directly linked to the classical morphology-
391 based biogenicity criteria. To date, this analytical approach has not been applied to abiotic vesicle

392 experiments although it is clearly necessary to provide control data for these kinds of microfossil
393 investigations.

394



395

396 *Figure 7. Comparison between purported microfossils and vesicle aggregates. Panels a-c are published*
397 *micrographs of controversial microfossils (a - Westall et al. (2001), b - Wacey et al. (2012), c - Wacey*
398 *et al. (2011)), d is a published micrograph of a biomorph from Cosmidis and Templeton (2016) formed*
399 *from sulfur and yeast extract, and panels e-h are micrographs of vesicles that were produced during*
400 *this study. All scale bars are 10 μm .*

401

402 *Comparison with other organic biomorphs*

403 Previous work from Cosmidis and Templeton⁵⁵ investigating the potential formation of carbon/sulfur-
404 based biomorphs produced a variety of spheroidal and filamentous structures (Fig. 7d) which
405 resemble the morphologies of filamentous vesicle solutions (Fig. 7h). This highlights the potential for
406 abiotic organic compounds from multiple sources to form microstructures that mimic microfossils.
407 The authors in this case call for ‘new caution’ in interpreting putative microfossils in the rock record⁵⁵.
408 In a recent study Nims et al.⁷¹ produced biomorphs containing sulphur and organic carbon which were
409 morphologically reminiscent of the filamentous sulphur-oxidizing bacteria *Thiothrix* containing
410 intracellular sulphur globules. Future isotopic analyses performed on these biomorphs could provide
411 some initial control data for sulphur and carbon containing microfossil interpretation. More
412 experimental work of this kind is imperative for our interpretation of microstructures in the rock

413 record. In another recent study Criouet et al. (2021)⁷² showed experimentally that under diagenetic
414 conditions RNA molecules mixed with quartz and water will create spheroidal organic microstructures
415 that mimic microorganisms such as Staphylococcus or Thermococcales. With these types of studies,
416 we are beginning to see only the tip of the iceberg of the potential for organic biomorphs to obscure
417 the rock record.

418

419 *Relevance for biogenicity tests*

420 Recently it has been suggested that the development of a strong set of criteria for determining the
421 biogenicity of microfossils is crucial to our understanding of the Earth's early biosphere⁷³. Clearly the
422 design of such criteria necessitates knowledge of the potential for abiotic structures to obscure our
423 interpretations. These microstructures may provide answers to questions that currently overshadow
424 uninterpreted signatures which have been observed in rock samples. It is essential that we understand
425 the formation and preservation of these structures particularly following diagenetic processes
426 representative of those experienced by relevant geological materials. Without the requisite control
427 data, we may continue to struggle through ambiguity. Understanding the composition of these non-
428 living structures would not only improve our interpretation of potential microfossils, but it could also
429 shed light on the environment in which abiotic structures were formed, further adding to our
430 knowledge of Earth's history. Furthermore, these biomorphs may be particularly beneficial for the
431 analysis of potential biosignatures on other planets where life may have started but perhaps did not
432 evolve in complexity. This is critical for the development of strategies for life-detection and
433 instruments for future space missions⁷³⁻⁷⁵. With sample return from Mars likely to be realised in the
434 not too distant future, it is essential that we are prepared to provide the best possible interpretation
435 of any proposed biosignatures that we identify during investigations here on Earth.

436

437 **Conclusions**

438 The current study presents a direct link between the origin of life and microfossil interpretation. We
439 have shown that lipid vesicles, representative of the first stages of cell membrane development at the
440 emergence of life, are capable of satisfying morphology criteria applied to traces of life from the early
441 Earth, and the development of advanced statistical approaches is required to pinpoint potential
442 differences which may enable us to distinguish between biogenic and abiogenic structures. It has been
443 suggested that the preserved remnants of prebiotic chemistry could potentially satisfy many of the
444 widely accepted biogenicity criteria currently applied to possible microfossils⁷⁵. The work presented
445 here represents a miniscule fragment of all possible prebiotic biomorphs which could be observed in
446 geological samples from the early Earth and potentially elsewhere in the Solar System. These

447 biomorphs could also inform on potential fossilised signatures of life's emergence which could be
448 observed in either terrestrial or extra-terrestrial samples – protobiosignatures. A significant effort is
449 required to determine the full range of possible prebiotic biomorphs which could affect our
450 interpretation of the earliest rock record including advanced physical, chemical, and statistical
451 analytical approaches.

452

453 **Author contributions**

454 Conceptualisation, S.F.J. and M.A.vZ.; Methodology, S.F.J, M.A.vZ., and J.R.; Experiments and analysis,
455 S.F.J., M.A.vZ., and J.R.; Writing—original draft, S.F.J.; Writing—review & editing, S.F.J., M.A.vZ., J.R.,
456 Z.M., and N.L.; Project administration, S.F.J.; Funding acquisition, S.F.J, M.A.vZ., Z.M., and N.L.. All
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458

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