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1 **Bubble functionalization in flotation process improve microalgae harvesting**

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32 **Abstract**

33 Microalgae are a promising resource for biofuel production, although the lack of effective harvesting  
34 techniques limits their industrial use. In this context, flotation, and in particular dissolved air flotation  
35 (DAF), is an interesting separation technique that could drastically reduce harvesting costs and make  
36 biofuel-production systems more economically viable. But because of the repulsive interaction  
37 between cells and bubbles in water, the efficiency of this technique can be limited. To solve this  
38 problem, we propose here an original DAF process where bubbles are functionalized with a bio-  
39 sourced polymer able to specifically bind to the surface of cells, chitosan. In a first part, we modify  
40 chitosan by adding hydrophobic groups on its backbone to obtain an amphiphilic molecule, PO-  
41 chitosan, able to assemble at the surface of bubbles. Then, using a recently developed technique  
42 based on atomic force microscopy (AFM) combined with microfluidics, we probe the interactions  
43 between PO-chitosan coated bubbles and cells at the molecular scale; results show an enhanced  
44 adhesion of functionalized bubbles to cells (from 3.5 to 12.8 nN) that is pH-dependent. Separation  
45 efficiencies obtained in flotation experiments with functionalized bubbles are in line with AFM data,  
46 and a microalgae separation efficiency of approximately 60% could be reached in a single step. In  
47 addition, we also found that PO-chitosan could be used efficiently as a flocculant (nearly 100% of  
48 cells removed), and in this case AFM experiments revealed that the flocculation mechanism is based  
49 on hydrophobic interactions between cells and PO-chitosan. Altogether, this comprehensive study  
50 shows the interest of PO-chitosan to harvest cells in flotation or flocculation/flotation processes.

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66 **Keywords:** Flotation, Flocculation, Functionalized bubbles, Chitosan, Atomic force microscopy,  
67 Microalgae

## 68 1. Introduction

69 Microalgae are photosynthetic microorganisms capable of capturing sunlight and converting  
70 carbon dioxide into value-added products such as biofuels, dietary products and animal feed. [1]. For  
71 biofuel production, microalgae are currently considered the most promising biomass due to their  
72 many advantages over terrestrial plants, such as rapid growth, high capacity to accumulate lipids  
73 under certain conditions and the possibility of growing them on non-arable land [2]. Despite these  
74 advantages, broad commercialization of microalgae-sourced biodiesel has been restrained due to the  
75 high costs involved in production processes. Basically, biofuel production from microalgae can be  
76 divided into the following major steps: cultivation, harvesting, extraction and down-stream processes  
77 [3]. The most expensive of these steps is the harvesting of microalgae; as they grow at low  
78 concentration (0.3–3 g/L), large volumes of water need to be treated to recover small quantities of  
79 biomass [4]. Although the choice of microalgae harvesting technique depends largely on the  
80 microalgae species and the desired end product, the most commonly used techniques are  
81 centrifugation, filtration and sedimentation [5]. These methods however are generally associated  
82 with a low efficiency, high capital costs and important energy and/or chemicals consumptions. For  
83 example, centrifugation requires a high energy input (up to 8 kWh/m<sup>3</sup> of microalgae, [6]) which  
84 represents a huge cost for largescale processing, and may also damage cells due to the high shear  
85 forces, resulting in a significant loss of the products of interest [1]. Likewise permeable membranes  
86 used for filtration are easily clogged by small microalgae [7], which also leads to important processing  
87 costs and material costs.

88 In this context, flotation could be an interesting alternative harvesting technique as it is a  
89 proven technology to efficiently capture small particles in an aqueous solution using air bubbles. In  
90 this way, it takes advantage of the natural characteristics of microalgae, namely a relatively low  
91 density and a tendency to self-flotation [8]. In addition, because it is a relatively rapid operation, with  
92 low space requirements, high flexibility and moderate operational costs, flotation technique has the  
93 potential to overcome the bottleneck of feasible microalgal biofuel production [9]. Indeed, when  
94 combined to a flocculation step, the energy demand reported can be as low as 1.5 kWh/m<sup>3</sup> [10].  
95 However, its efficient use for microalgae harvesting is still challenging as cells are usually negatively  
96 charged. The surface of air bubbles being also negatively charged in water, [11] they repel each  
97 other preventing adhesion and thus capture and flotation. To improve flotation efficiency, adding a  
98 flocculation step prior to flotation can be a good solution. Synthetic flocculants added to the  
99 microalgal suspension aggregate cells into large flocs that can be easily captured by the bubbles [12].  
100 However, contamination is a major issue in this technique as flocculants at the end of the process  
101 end up in the harvested biomass and can have an important impact on the final quality of the  
102 products [9]. To avoid this problem, natural flocculation is a preferred alternative. So far, two types  
103 of natural flocculation mechanisms have been identified: auto-flocculation, where flocculation is  
104 triggered by a molecule or precipitate that forms naturally in the culture medium, and bio-  
105 flocculation, where a molecule produced by cells is directly responsible for flocculation [13]. But  
106 because natural flocculation can be difficult to control or trigger in industrial processes, many studies  
107 have showed the interest of using bio-flocculants like biopolymers either directly extracted from  
108 other organisms like natural polysaccharides, or modified by various means to control the functional  
109 chemical groups they present and induce natural flocculation [14–17]. The most popular biopolymer  
110 used for microalgae harvesting is with no doubt chitosan. Chitosan is a cationic polyelectrolyte at pH  
111 lower than its pKa (6.5) obtained by deacetylation of chitin. After cellulose, it is the second most  
112 abundant natural polymer on earth [18,19]. Moreover as chitin-like polysaccharides are naturally  
113 present in the cell wall of several microalgae species [20], chitosan does not contaminate the  
114 harvested biomass. To understand its flocculation mechanism, our team recently performed atomic

115 force microscopy (AFM) experiments to probe the interactions between chitosan and cells. AFM, first  
116 developed in 1986, is a powerful tool that can be used to study microalgae cells at the nanoscale and  
117 characterize their interactions with their environment [21]. The results obtained in this study showed  
118 that at low pH, chitosan is able to form specific interactions with polymers present at the surface of  
119 cells, in this case, cells of *Chlorella vulgaris*, while at higher pH, chitosan forms a precipitate in which  
120 cells get entrapped [17].

121 Another possibility to improve the efficiency of flotation for microalgae harvesting that has  
122 been explored is to modify the surface of the bubbles. The principal example of such a strategy was  
123 provided by Henderson's team, who modified the surface of the bubbles with positively charged  
124 polymers, thereby changing the charge of the bubbles and making interaction with the cells  
125 attractive. Using this strategy named Posi-DAF (positive dissolved air flotation), the authors could  
126 obtain a maximum separation efficiency of 97%, 54% and 89% in the case of *Melosira aeruginosa*, *C.*  
127 *vulgaris* and *Asterionella formosa* cells respectively. Here in this work, we also propose a bubble-  
128 modification strategy, based on the recent findings that we generated on the mechanism of  
129 interaction of chitosan with cells [17]. The hypothesis is that since chitosan is able to bind specifically  
130 to microalgae cells at low pH, if we functionalize it at the surface of bubbles, then flotation  
131 separation could be efficient without the need of a flocculation step. Removing this step in a large-  
132 scale production system could result in reduced costs, reduced harvesting time, and could represent  
133 an important step forward for the use of microalgae for biofuel production. This is what is presented  
134 in this study, and for that we worked with a biotechnologically-relevant freshwater microalgae  
135 species, *C. vulgaris*. The first step of this work was to modify chitosan so it could be functionalized at  
136 the surface of bubbles, by adding hydrophobic groups on its hydrophilic backbone. Then, using a  
137 recently developed approach based on FluidFM technology, which combines AFM and microfluidics,  
138 we could probe the interactions between functionalized bubbles and cells at the molecular scale, and  
139 this way understand the mechanism involved in this interaction [22]. Finally, the effectiveness of this  
140 original flotation process in different experimental conditions was determined. But as we were  
141 investigating the interacting behavior of this chitosan-based molecule, we also found that it could be  
142 successfully used as a flocculant; AFM experiments in this case allow understanding how the  
143 modifications made on chitosan affected the physico-chemical basis of its interactions with cells.  
144 Altogether, this work has led to the development of an original flotation process based on  
145 functionalized bubbles with a modified chitosan molecule, which can also serve as a flocculant  
146 depending on the application and needs. Finally the AFM experiments performed at the molecular  
147 scale could highlight the mechanisms at play, thereby giving a full understanding of the interaction  
148 mechanisms involved in both cases.

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## 158 **2. Materials and methods**

### 159 **2.1. Chemicals.**

160 Chemicals for the synthesis of alkyl-chitosan derivatives were the following: chitosan (from shrimp,  
161 practical grade,  $\geq 75\%$  degree of deacetylation, C3646), octanal (O5608), sodium hydroxide (S0899),  
162 sodium cyanoborohydride reagent grade 95% (156159), Deuterium chloride solution (543047) were  
163 purchased from Sigma-Aldrich as well as glacial acetic acid 99.5% (W200611) and ethanol 96%  
164 (1.59010) and used as received.

### 165 **2.2. Synthesis and characterization of polyoctyl chitosan (PO-chitosan).**

166 The N-octyl-chitosan derivatives were obtained by reductive amination following a procedure  
167 previously described in the literature [23–26]. In brief, 6 g of chitosan were dissolved in 450 mL of 0.2  
168 M acetic acid (AcOH) to which was added 180 mL of ethanol after complete dissolution. The pH was  
169 adjusted to 6 with 4 M of NaOH to prevent macromolecule precipitation. A solution of octanal (target  
170 alkylation level of 10%) in 40% of ethanol was added using a 1:3 ratio prior to adding an excess of  
171 sodium cyanoborohydride ( $\text{NaBH}_3\text{CN}$ ) (3:1 mole ratio per glucosamine monomer). After stirring for  
172 24h at room temperature, the pH of the reaction mixture was adjusted to 7-8 using a solution of 4 M  
173 of NaOH. The precipitate was collected by centrifugation for 5 min at 6000 rpm at 4°C and was then  
174 thoroughly washed with ethanol/water mixture at least 5 times with increasing ethanol  
175 concentration from 40% (v/v) to 100% (v/v) before drying until constant weight. NMR spectroscopy  
176 was used to characterize both chitosan and N-octyl-chitosan derivatives produced to determine the  
177 degree of substitution (DS). The NMR spectra were performed on a Bruker Advance spectrometer  
178 (Bruker, Switzerland) in  $\text{D}_2\text{O}$ -DCl (pH around 4) at a resonance frequency of 400.13 MHz and 70°C on  
179 the starting material and on the final product. The degree of substitution was calculated from NMR  
180 spectra as previously described elsewhere [23]. Integration of the anomeric protons and acetyl  
181 groups were obtained using the TOPSPIN 4.0.8 software (Bruker, Switzerland) and gave an  
182 acetylation degree of 20% (consistent with the starting material), 12% of octylated monomers, and  
183 68% of free amine monomers.

### 184 **2.3. Microalgae strain and culture.**

185 The green freshwater microalgae *Chlorella vulgaris* strain CCAP 211/11B (Culture Collection of Algae  
186 and Protozoa, Scotland, UK) was cultivated in sterile conditions in Wright's cryptophyte (WC)  
187 medium prepared with deionized water, as previously described [17]. Cells were cultivated at 20°C,  
188 under 120 rpm agitation, in an incubator equipped with white neon light tubes providing illumination  
189 of approximately  $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  with a photoperiod of 18h light: 6h dark. Exponential  
190 phase experiments were performed with 7-day batch cultures, while stationary phase and salinity  
191 stress condition (0.1M NaCl) experiments were performed with 21-day batch cultures.

### 192 **2.4. Roughness analyses.**

193 Roughness analyses were performed on PO-chitosan immobilized on glass slides. PO-chitosan was  
194 functionalized at the surface of glass slides using spin-coating, according to a procedure described in  
195 Demir *et al.* 2020 [17]. Briefly, 2.5 g/L PO-chitosan (pH around 2 adjusted with HCl) solution was  
196 deposited on a clean glass slide and spin-coated at 1000 rpm for 3 min. The glass slides were then  
197 dried in an incubator at 37°C overnight before use. Height images of the PO-chitosan surfaces were

198 recorded in PBS at pH 6, 7.4 and 9 using contact mode available on the Nanowizard III AFM (Bruker,  
199 USA), and MSCT cantilevers (Bruker, nominal spring constant of 0.01 N/m). Images were recorded  
200 with a resolution of 256 x 256 pixels using an applied force < 1 nN. In all cases the cantilevers spring  
201 constants were determined by the thermal noise method prior to imaging [27]. The height images  
202 obtained were then analyzed using the Data Processing software (Bruker, USA) to determine the  
203 arithmetic average roughness (Ra) of 6 different areas of 25  $\mu\text{m}^2$  (5  $\mu\text{m}$  x 5  $\mu\text{m}$ ) for each sample.

## 204 **2.5. Flocculation and flotation experiments.**

205 Flocculation and flotation separation of *C. vulgaris* was performed in a dissolved air flotation (DAF)  
206 homebuilt flotation device, described elsewhere [28]. Briefly, the depressurization at atmospheric  
207 pressure of water saturated by air at 6 bars induced the formation of bubbles. Water free of algae  
208 was pressurized for 30 min before injection into the jars. The injection was controlled by solenoid  
209 valves and a volume of pressurized water was added to each beaker sample. Two types of  
210 experiments were conducted, repeated 3 times for each condition with cells coming from 2  
211 independent cultures:

- 212 • **Flocculation:** *C. vulgaris* cells were cultured during 7 days until they reached mid-exponential  
213 phase. Then 100 mL of cell suspension was directly poured into the test-jars with an initial optical  
214 density (OD) at 750 nm of 0.8. Flocculants, chitosan and PO-chitosan, were directly added (final  
215 concentration of 10, 15 and 20 mg/L for chitosan and of 12, 17, 22, 25, 30, 40 and 60 mg/L for  
216 PO-chitosan) to the suspension, which was stirred at 100 rpm for 20 minutes to homogenize and  
217 left to settle for 30 minutes. OD at 750nm of the suspension was measured afterwards to  
218 calculate flocculation efficiency.
- 219 • **Flotation:** *C. vulgaris* cells were cultured during 7 days until they reached mid-exponential phase.  
220 Then, 100 mL of cell suspension was directly poured into the test-jars with an initial OD<sub>750</sub> of 0.8.  
221 PO-chitosan mixed with water was directly added to the pressurization tank (final concentration  
222 of 30, 25 and 20 mg/L); the mix was then pressurized during 30 minutes at 6 bars. Following this,  
223 depressurization at atmospheric pressure of the water-PO-chitosan mix saturated by air was  
224 performed to inject functionalized microbubbles into each flotation beaker (bubble volume of 20,  
225 50, 80 and 100 mL). The algal suspension was retrieved from the bottom of the test-jars: 30 mL  
226 were used for quantifying flotation efficiency.

227 For both types of experiments, the optical density of the withdrawn microalgae suspension (OD<sub>f</sub>) was  
228 measured and compared to the optical density of the microalgae suspension measured before the  
229 experiments (OD<sub>i</sub>), taking the initial and final volumes into account (V<sub>i</sub> and V<sub>f</sub>). The flotation efficiency  
230 (E) was calculated according to the following equation 1.

231

$$E = \frac{OD_i \cdot V_i - OD_f \cdot V_f}{OD_i \cdot V_i} \quad (1)$$

## 232 **2.6. Zeta Potential Experiments.**

233 The global electrical properties of *C. vulgaris* cell surface at pH 6, 7.4 and 9 were assessed by  
234 measuring the electrophoretic mobility with an automated laser zetameter (Zetasizer NanoZS,  
235 Malvern Instruments). To this end, microalgae were harvested by centrifugation (3000 rpm, 3 min),  
236 washed two times in PBS at a pH of 6, 7.4 or 9, and resuspended in the same solution at a final  
237 concentration of  $1.5 \times 10^6$  cell/mL. For each condition, analysis were performed in triplicate.

## 238 **2.7. Granulometry analysis.**

239 Particle size distributions of both chitosan and PO-chitosan were determined using a Mastersizer  
240 (Malvern Instruments, UK). For that, PO-chitosan was dissolved in water with a pH around 2 (with  
241 HCl) and stirred for 1 week at final concentrations of 2.5 g/L, 1 g/L and 0.5 g/L. The refractive index  
242 used for micelles was of 1.350. The results are presented as an average number obtained from 3  
243 measurements.

## 244 **2.8. Force spectroscopy experiments using FluidFM technology.**

245 Force spectroscopy experiments were conducted using a NanoWizard III AFM (Bruker, USA),  
246 equipped with FluidFM technology (Cytosurge AG, Switzerland). In each case, experiments were  
247 performed in PBS at pH 6, using micropipette probes with an aperture of 2  $\mu\text{m}$  (spring constant of  
248 0.3, and 4 N/m, Cytosurge AG, Switzerland). First, PBS at a pH of 6 was used to fill the probe reservoir  
249 (5  $\mu\text{L}$ ); by applying an overpressure (100 mBar) the PBS then filled the entire cantilever microchannel.  
250 The probe was then immersed in PBS and calibrated using the thermal noise method prior to  
251 measurement [27]. A single *C. vulgaris* cell was then aspirated from the surface of the Petri dish by  
252 approaching the FluidFM probe and applying a negative pressure (-200 mBar). The presence of the  
253 cell on the probe was verified by optical microscopy. The cell probe was then used to measure the  
254 interactions with PO-chitosan immobilized on glass slides. Interactions between single *C. vulgaris*  
255 cells aspirated at the aperture of FluidFM cantilevers and PO-chitosan were recorded at pH 6, 7.4 and  
256 9 at a constant applied force of 1 nN, force curves were recorded with a z-range of up to 2  $\mu\text{m}$  and a  
257 constant retraction speed of 2.0  $\mu\text{m/s}$  to 4  $\mu\text{m/s}$ . Data were analyzed using the Data Processing  
258 software from Bruker. Adhesion forces were obtained by calculating the maximum adhesion force for  
259 each retract curves. Results were recorded on ten different cells coming from at least two  
260 independent cultures.

## 261 **2.9. Bubble formation and functionalization using FluidFM.**

262 Air-bubbles were formed using FluidFM as described in Demir *et al.* [22], using a Nanowizard III AFM  
263 (Bruker, USA), equipped with FluidFM technology (Cytosurge AG, Switzerland). Experiments were  
264 performed in PBS, using microfluidic micropipette probes (FluidFM cantilevers) with an aperture of 8  
265  $\mu\text{m}$  (spring constant of 0.3 and 2 N/m, Cytosurge AG, Switzerland). Briefly, hydrophobized FluidFM  
266 cantilevers were first filled with air and immersed in PBS. A single bubble was then formed at the  
267 aperture of the cantilevers by applying a positive pressure (200 mbar) inside the cantilever thanks to  
268 the pressure controller to which it is connected. To produce functionalized bubbles, the FluidFM  
269 cantilever was immersed in a solution of 2 mg/L of PO-chitosan. This solution was aspirated inside  
270 the cantilever by gradually decreasing the pressure from 0 mbar to -200 mbar. After that the FluidFM  
271 cantilever containing the surfactant solution was immersed in PBS buffer without surfactants. By  
272 increasing the pressure to 150 mbar, the surfactant solution was then locally dispersed in the buffer  
273 and a bubble was formed: the surfactant then assembled at the surface of the produced bubble.  
274 Interactions between PO-chitosan coated bubble produced at the aperture of FluidFM and single *C.*  
275 *vulgaris* cells were recorded at pH 6, 7.4 and 9 at a constant applied force of 1 nN, force curves were  
276 recorded with a z-range of up to 2  $\mu\text{m}$  and a constant retraction speed up to 4  $\mu\text{m/s}$ .

## 277 **2.10. Statistical analysis.**

278 Experimental results represent the mean  $\pm$  standard deviation (SD) of at least three replicates. For  
279 each experiments, the number of replicates is indicated in the results and discussion section. For  
280 large samples, student t-test was used to assess the difference observed in the results. For small  
281 samples (less than 20) Mann and Whitney test was used to assess the difference. The differences  
282 were considered significantly at  $p < 0.05$ .

283



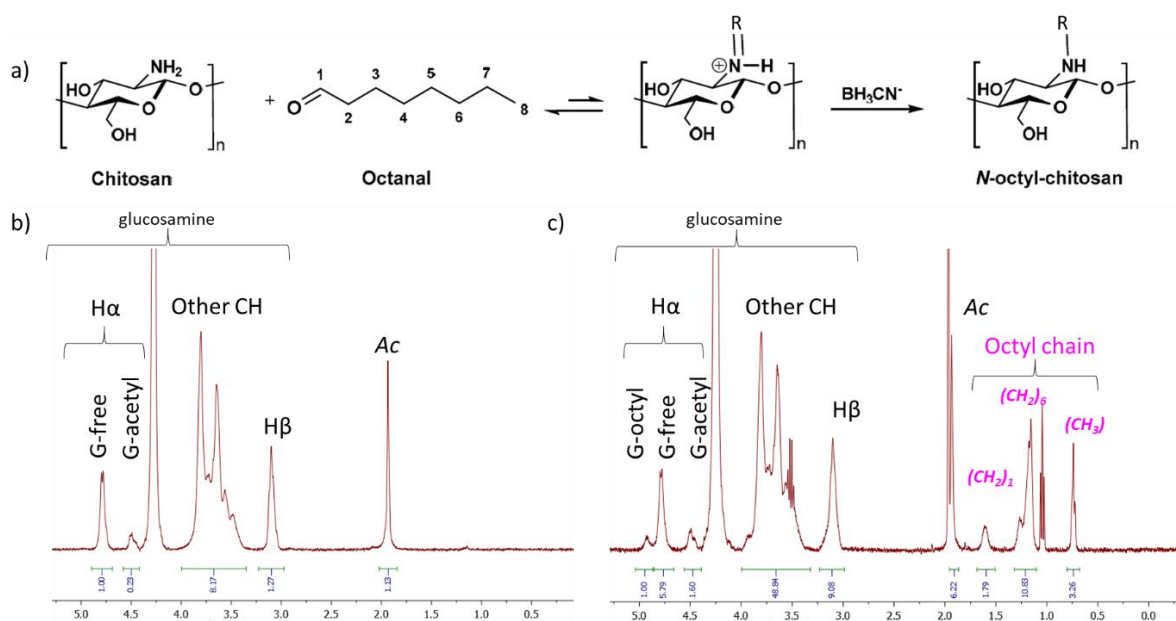
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### 286 3. Results and Discussion

#### 287 3.1. Synthesis of PO-chitosan

288 The first step of this study consisted in the synthesis of PO-chitosan using the reductive  
289 amination reaction illustrated in Figure 1a. Such reaction preserves the number of basic nitrogens  
290 and can be performed under mild conditions that do not modify the chitosan molecule itself (degree  
291 of acetylation and/or polymerization degree) as described elsewhere [24]. Octanal was chosen as a  
292 precursor of the hydrophobic alkyl groups, this way the amphiphilic character of the target molecule  
293 PO-chitosan can be reached without the complete alkylation of all glucosamine monomers and  
294 indeed a 10% stoichiometric ratio is sufficient. Thus, some of the primary amino groups of chitosan  
295 undergo a Schiff reaction with octanal to yield the corresponding aldimines, which are then  
296 converted to alkyl derivatives by reduction with  $\text{NaBH}_3\text{CN}$ .  $^1\text{H-NMR}$  spectroscopy in  $\text{D}_2\text{O}/\text{DCI}$  ( $\text{pH} \sim 4$ )  
297 at  $70^\circ\text{C}$  was used to characterize both chitosan and PO-chitosan and determine the degree of  
298 substitution (DS) of the amine functions by the octyl chains. The  $^1\text{H-NMR}$  spectra of initial chitosan  
299 and PO-chitosan are presented in Figure 1b and c respectively. The octyl chain in PO-chitosan can be  
300 easily identified by the signals in the 0.7 – 1.7 ppm region. Thus, the signal at 0.7 ppm was attributed  
301 to the terminal  $-\text{CH}_3$  while those at 1.6 ppm and the multiplet at 1.1-1.3ppm, to respectively the -



302  $\text{CH}_2$  group linked to the N atom and the core  $\text{CH}_2$  of the octyl chain.

303 **Figure 1. Synthesis of PO-chitosan.** a) Synthesis of PO-chitosan by alkylation, b)  $^1\text{H-NMR}$  spectra obtained for  
304 initial chitosan, c)  $^1\text{H-NMR}$  spectra obtained for PO-chitosan. The DS obtained for PO-chitosan is of 12%.

305 The degree of substitution was calculated as previously described [23], by examining the relative  
306 integration of the anomeric protons H $\alpha$ , using the assignation previously reported [23–25].

307 \* at 4.54 ppm, the acetylglucosamine unit,

308 \* at 4.80 ppm, the unsubstituted glucosamine unit

309 \* at 4.94 ppm, the monosubstituted glucosamine unit.

310

311 It was found to be of 12% (Figure 1c), meaning that 12% of the amine functions of chitosan have  
312 been modified with octanal molecules. This number is close to the targeted degree of substitution  
313 (10%) and to what was found by Mati-Baouche *et al.* who described the reaction in these specific  
314 conditions [24]. The relative number of N-acetylated glucosamine remained unchanged compared to  
315 the starting chitosan confirms the mildness of the reaction conditions used.

316

### 317 **3.2. Characterization of PO-Chitosan using atomic force microscopy**

318 PO-chitosan has already been characterized on the basis of its water resistance, rheological  
319 characteristic and bonding properties to wood and aluminum surfaces [24,25]. Here we  
320 characterized PO-chitosan on the basis of its surfactant properties, particle size, roughness and  
321 hydrophobicity, which are parameters important to then optimize the next experiments of this study  
322 (AFM, flocculation and flotation experiments). PO-chitosan has both hydrophilic (-NH<sub>2</sub> or -OH) and  
323 hydrophobic sites (alkyl chains, octanal), and thus possess amphiphilic properties, making it a  
324 surfactant. As for any surfactants, it should be able to decrease the surface tension of water with  
325 increasing concentration. Surface tension experiments were then performed, the results are  
326 presented in Supplementary Figure 1. They show that with increasing concentration of PO-chitosan,  
327 the surface tension of water decreases from approximately 72 to 62 mN/m for a PO-chitosan  
328 concentration of 2.5 g/L. This decrease as important as it can be with other types of surfactants, but  
329 this can be explained by the degree of substitution of the molecule, which is of 12%. This means that  
330 only 12% of the amine functions of chitosan have been modified with hydrophobic octanal  
331 molecules, thus the hydrophobic part of the molecule may not be large enough to change in an  
332 important manner the surface tension of water. However, in order to be able to dissolve the  
333 molecule in water, there needs to be a balance between the hydrophobic and hydrophilic groups. For  
334 instance, for low molecular weight chitosan, even with a substitution degree of 10%, the resulting  
335 molecule is water insoluble. Whereas, for high molecular weight chitosan we are limited with low  
336 alkylation level (10-15 %) because as the alkylation level increases, water solubility of PO-chitosan  
337 decreases, and we need water soluble compounds to use for the next experiments. To verify we can  
338 completely dissolve PO-chitosan in water, we measured the particle size of both initial chitosan and  
339 PO-chitosan in water using granulometry. The size distribution graphs obtained are shown in  
340 Supplementary Figure 1b and c respectively (concentration of 2.5 g/L). They both show a similar  
341 pattern which means that the addition of octanal does not modify the size of chitosan significantly  
342 (high molecular weight).

343

344 Moreover, we also measured the turbidity of chitosan and PO-chitosan solutions at different  
345 concentrations (2.5, 1 and 0.5 g/L): the obtained turbidities are of 4.3, 3.2 and 3.5 NTU respectively.  
346 This means that solutions are clear (NTU < 5 corresponds to clear water). Thus both size  
347 measurements and turbidity experiments prove that we are able to dissolve PO-chitosan in water.  
348 Moreover, based on the literature we know that chitosan and PO-chitosan behave differently. For  
349 example, rheological analysis show that alkyl-chitosan solutions are non-Newtonian fluids, since the  
350 viscosity decreases with increasing shear rate whereas initial chitosan shows a Newtonian behavior  
351 [23–25]. Further, Desbrieres *et al.* highlighted that addition of octanal or increase in the DS, is linked  
352 with the increase in viscosity of PO-chitosan since the intermolecular hydrophobic interaction is a key  
353 element in physico-chemical (rheological) properties of the modified chitosan. The higher the  
354 hydrophobic properties (the length of the alkyl chain or degree of substitution) of macromolecular  
355 chain the larger the gap to the Newtonian behavior [25]. Thus, more analysis needs to be performed

356 on PO-chitosan to understand the differences with chitosan, such as roughness and hydrophobicity  
 357 measurements.

358  
 359 Regarding the hydrophobic properties of PO-chitosan, it has been found that pH has an  
 360 influence on the hydrophilic and hydrophobic balance of the molecule due to the ability of  $-NH_2$   
 361 functions (hydrophilic part) to be ionized in acidic conditions [23]. We thus measured the  
 362 hydrophobicity of PO-chitosan at different pH (pH 6, 7.4 and 9) relevant for flocculation or flotation  
 363 processes for *C. vulgaris* cells, using a method recently developed in our team based on the  
 364 interactions between bubbles produced by FluidFM and samples [22,29]. Air bubbles in water  
 365 behaving like hydrophobic surfaces, by measuring their direct interactions with surfaces it is possible  
 366 to determine the hydrophobic properties of the samples in terms of adhesion force, and further  
 367 convert these forces into water contact angles (WCA) [22]. Using WCAs values we can then compare  
 368 our data directly to the ones available in the literature. To perform these experiments, PO-chitosan  
 369 was immobilized on glass slides by spin coating and their interaction with bubble were measured in  
 370 PBS buffer at pH 6, the pH generally used for chitosan induced flocculation, pH 7.4, the optimum pH  
 371 for *C. vulgaris* growth, or pH 9 which corresponds to the pH *C. vulgaris* cultures reach after 7 days. A  
 372 schematic representation of these measurements is presented in Figure 2a. The adhesion force  
 373 histograms obtained at pH 6, 7.4 and 9 are presented in Figure 2b, c and d respectively. In each case  
 374 the force curves obtained show a single peak occurring at the contact point (inset in Figure 2b, c and  
 375 d), which is characteristic of non-specific interactions such as hydrophobic interactions [30]. On each  
 376 force curve obtained, the adhesion force is then quantified by measuring the height of this adhesion  
 377 peak, which corresponds to the force needed to break the interaction between the bubble and the  
 378 sample. This force reflects the degree of hydrophobicity of the sample, the stronger the adhesion,  
 379 the higher the hydrophobicity. In the case of pH 6, the average adhesion force is of  $66.7 \pm 13.9$  nN  
 380 (Figure 2b,  $n = 3125$  force curves obtained on 5 different measurements). While this value stays  
 381 similar at a pH of 7.4 ( $64.6 \pm 20.3$  nN, Figure 2c,  $n = 1977$  force curves obtained on 5 different  
 382 measurements), it decreases to  $46.5 \pm 15.9$  nN at pH 9 (Figure 2d,  $n = 1454$  force curves obtained on  
 383 5 different measurements). Even though the average adhesion values at pH 6 ( $66.7 \pm 13.9$  nN) and  
 384 7.4 ( $64.6 \pm 20.3$  nN) are close to each other, statistical analysis shows that they are significantly  
 385 different ( $p$ -value of 0.05, unpaired student test). The conversion of these adhesion values into WCAs  
 386 gives the results presented in Table 1, which show that indeed, pH has an effect on the  
 387 hydrophobicity of the molecule, as the WCA decreases with increasing pH values. The important  
 388 point to note as well in this case is that initial chitosan is completely hydrophilic (WCA of 0) and does  
 389 not interact with bubbles (Supplementary Figure 2) whatever the pH considered; it is the  
 390 modifications made on the molecule and the addition of octanal that confers amphiphilic properties  
 391 to PO-chitosan.

392  
 393 **Table 1. Hydrophobic properties of PO-chitosan at different pH.** Adhesion values obtained by FluidFM and  
 394 corresponding water contact angle (WCA) of chitosan and of PO-chitosan surfaces at pH 6, 7.4 and 9.

Sample	pH	Adhesion value (nN)	WCA (°)
Chitosan	6	0	~ 0
	7.4	0	~ 0
	9	0	~ 0
PO-chitosan	6	$66.7 \pm 13.9$	48.7
	7.4	$64.6 \pm 20.3$	48.3

395

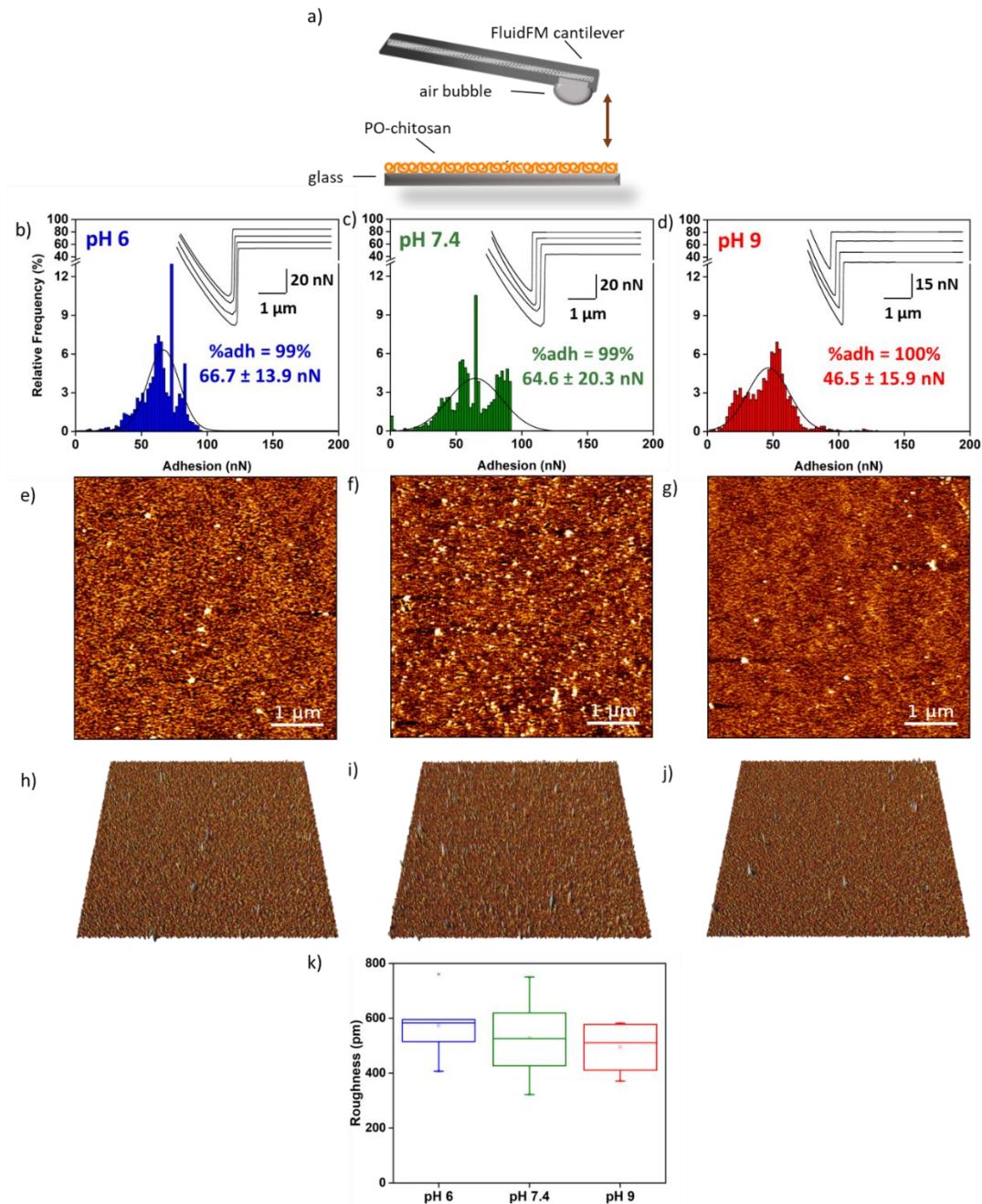
9

46.5 ± 15.9

44.6

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396 Previous studies have showed that chitosan, at elevated pH, precipitates into the medium  
 397 [16,17]. This precipitation was visible when we imaged in a previous study chitosan coated surfaces  
 398 using AFM, where aggregates of chitosan formed on the surface, resulting in an increased roughness  
 399 ( $13 \pm 5$  nm) compared to low pH ( $0.6 \pm 0.1$  nm) [17]. To check whether PO-chitosan behaves like  
 400 chitosan at high pH, we further characterized it by imaging PO-chitosan surfaces at pH 6, 7.4 and 9  
 401 using AFM in contact mode. The height images obtained are presented in Figure 2e, f and g  
 402 respectively, they show a similar topography in all cases, with no aggregates present on the surface.  
 403 The quantification of the surface roughness in each case gave similar values (box plot in Figure 2k),  
 404 with a roughness of  $574.0 \pm 105.7$  pm at pH 6, of  $528.1 \pm 144.8$  pm at pH 7.4 and of  $494.1 \pm 82.2$  pm



405 at pH 9. Non-parametric statistical tests (Mann and Whitney test) showed that these values indeed  
 406 are not significantly different. These results confirm the observations from the height images,  
 407 whatever the pH, PO-chitosan surfaces are homogeneous with no aggregates formed, meaning that  
 408 PO-chitosan does not precipitate at high pH like chitosan does.

409 **Figure 2: Characterization of PO-chitosan surface at different pH.** a) Schematic representation of bubble and  
410 PO-chitosan surface interaction. Adhesion force histogram obtained between bubble and PO-chitosan surface at  
411 b) pH 6 c) pH 7.4 and d) pH 9. AFM height images of PO-Chitosan surface at e) pH 6 (color scale = 4 nm) f) pH 7.4  
412 (color scale = 4 nm) and g) pH 9 (color scale = 4 nm) and their corresponding 3D AFM vertical deflection images  
413 h) pH 6 i) pH 7.4 and j) pH 9. k) Quantification of PO-chitosan surface roughness at different pH.

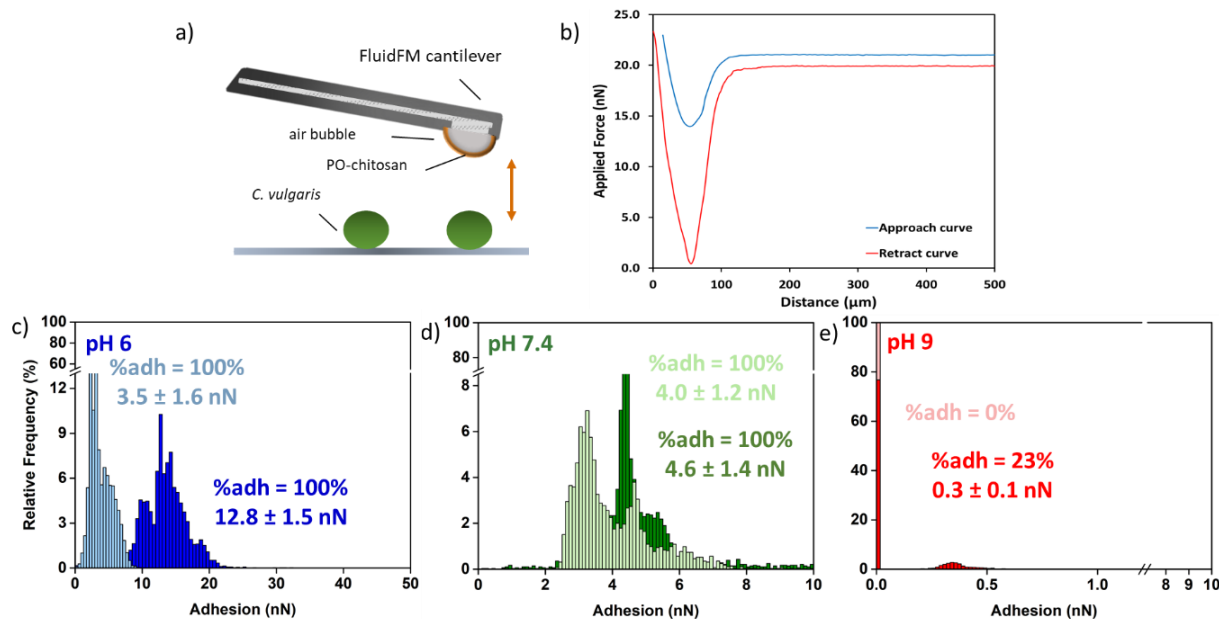
414 Thus, in summary, the modification of chitosan by addition of hydrophobic octanal molecules  
415 on its backbone, with a DS of 12%, made it amphiphilic as confirmed by surface tension experiments  
416 that showed a decrease in the surface tension. The new molecule PO-chitosan can also be  
417 completely dissolved in water, as confirmed by particle size and turbidity measurements. Then, using  
418 FluidFM experiments, we showed that the modifications made indeed changed the hydrophobic  
419 properties of the molecule, which are dependent on the pH, as described in the literature. Finally  
420 AFM measurements showed that PO-chitosan, unlike chitosan, does not precipitate at elevated pH.  
421 Now the next step of this study is to functionalize it at the surface of bubbles and determine if this  
422 functionalization allows a better adhesion of bubbles with cells.

### 423 3.3. PO-chitosan functionalized bubbles improve flotation efficiency

424 The characterization of PO-chitosan has showed that the molecule is indeed amphiphilic, able  
425 to act like a surfactant, thus we can use it to coat the surface of bubbles. The question is now to  
426 know if the presence of PO-chitosan on the surface of bubbles improves its adhesion to cells, and by  
427 which mechanism. To verify this point, we modified the surface of bubbles produced with FluidFM  
428 with PO-chitosan (concentration of 2 mg/L) using a protocol previously developed in our team [22].  
429 Briefly, for that, a solution containing PO-chitosan is first aspirated inside a FluidFM cantilever. The  
430 cantilever is then immersed in the petri dish containing cells: by applying a positive pressure, the PO-  
431 chitosan solution is released, a bubble is formed, and because PO-chitosan molecules are in close  
432 proximity of the bubble, they directly assemble at its surface. PO-chitosan coated bubbles can then  
433 be used to probe the direct interactions with *C. vulgaris* cells. The results are presented in Figure 3.  
434 Figure 3a is a schematic representation of the experimental set-up. In this case the retract force  
435 curves obtained showed a single retract peak at the contact point (red curve in Figure 3b) with an  
436 average adhesion force of  $12.8 \pm 1.5$  nN at pH 6 (Figure 3c in dark blue, n= 3603 force curves from 7  
437 different cells coming from 2 independent cultures). This interaction is 3.6 times higher than the one  
438 obtained between clean bubbles and cells (Figure 3c in light blue), meaning that indeed, the  
439 functionalization of the bubble surface with PO-chitosan enhances the direct interaction with *C.*  
440 *vulgaris* cells. Moreover, the approach force curve also shows a “jump-in” peak reflecting the fact  
441 that the PO-chitosan coated bubble gets suddenly attached to the *C. vulgaris* cell (Figure 3b, blue  
442 curve). This jump-in, as previous studies on bubble-hydrophobic surface interaction show [22], is  
443 most likely due to the long-range hydrophobic force that causes the disruption of the water film and  
444 the formation of the three phase contact (TPC) line. This is an important point. Indeed, when we  
445 characterized the interactions between chitosan and *C. vulgaris* cells in our previous study, the force  
446 curves obtained showed multiple peaks taking place away from the contact point, materializing the  
447 unfolding of polymers from the surface of cells upon retraction [17]. In theory, the hydrophobic parts  
448 of PO-chitosan should be inside the air bubble, while the rest of the chitosan molecule, which is  
449 hydrophilic, should be outside the bubble, available for interaction. Thus, we expected to obtain the  
450 same interactions with PO-chitosan coated bubbles as we had with chitosan alone. This is clearly not  
451 the case, and our hypothesis to explain this is that when the coated bubble contacts the cell, the  
452 specific interaction between the hydrophilic backbone of the chitosan and the cell can take place, but  
453 because this interaction is effective, the water film between the bubble and the cell breaks down,  
454 resulting in the formation of the TPC line. At this point, when the bubble probe is retracted from the

455 cell, the hydrophobic interaction becomes dominant over the specific interaction, and this is what we  
456 see on the force curve. The fact that this hydrophobic force is much higher in the case of PO-chitosan  
457 coated bubbles compared to clean bubbles can be explained by the first attractive specific  
458 interaction of the cells with the hydrophilic backbone of the chitosan present on the bubble surface.  
459 In addition, the formation of the TPC line increases the contact area between the bubble and the *C.*  
460 *vulgaris* cells, which increases the adhesion forces obtained. For example, in our previous study we  
461 already prove that there is a direct relationship between effective radius (thus the contact area) and  
462 hydrophobic forces. Meaning that increase in hydrophobicity enhances the effective radius between  
463 hydrophobic surfaces and bubble due to the TPC line formation thereby leading to higher adhesion  
464 forces [22].

465 We further repeated these experiments at pH 7.4 and 9 (Figure 3d and e). At pH 7.4, cells  
466 interact more with clean bubbles with an average adhesion force of  $4.0 \pm 1.2$  nN (Figure 3d, light  
467 green histogram,  $n= 2814$  force curves from 5 cells) compared to pH 6, which can be explained by  
468 some changes perhaps in the hydrophobicity of *C. vulgaris* cells surface at this pH. When bubbles are  
469 functionalized with PO-chitosan, the average force obtained is of  $4.6 \pm 1.4$  nN (Figure 3d, dark green  
470  $n= 2814$  force curves from 5 cells), thus almost 3 times less than at pH 6. In addition in this case, the  
471 “jump-in” peak on the approach curves was not visible anymore. This important decrease in the  
472 adhesion is most probably due to the decrease in the hydrophobicity of PO-chitosan molecule.  
473 Although this decrease is low, it has important consequences on the interactions with cells. At pH 9  
474 (Figure 3e), cells do not interact with clean bubbles (0% of adhesion, light red bar in Figure 3e) at all,  
475 while when bubbles are coated with PO-chitosan, the percentage of force curves showing retract  
476 adhesions is of 23%, with an average force of  $0.3 \pm 0.1$  nN (dark red histogram in Figure 3e,  $n=4419$   
477 from 7 cells coming from 2 independent cultures). In the case of chitosan, there was no interaction  
478 with cells at higher pH, but this was explained by the fact that chitosan precipitated at such pH  
479 values. The roughness measurements performed in the first part of this work showed that PO-  
480 chitosan does not precipitate like chitosan does at higher pH. Therefore, this lack of interaction is  
481 probably not related to PO-chitosan, but to the cell surface itself. Indeed, in this case, clean bubbles  
482 do not interact with cells, which means that at pH 9, the cell surface is completely hydrophilic. This  
483 can be explained by a change in the cell wall composition at higher pH [20], or by a change in the cell  
484 surface architecture where hydrophobic molecules at the surface of cells may be masked by other  
485 components [17]. Thus, the initial interaction between the hydrophilic chitosan backbone at the  
486 surface of bubbles still takes place, as proven by the low adhesions recorded. However, because the  
487 cell surface is hydrophilic, the liquid film between the bubbles and the cells cannot be broken,  
488 resulting in a weak adhesion force. These results are important because they provide insight into the  
489 molecular mechanism underlying the interactions of PO-chitosan bubbles with cells. While the  
490 interaction with PO-chitosan bubbles probably starts with a specific interaction between the chitosan  
491 molecules present at the surface of bubbles and cell surface polymers, hydrophobicity remains the  
492 main factor allowing then the contact between bubbles and cells.



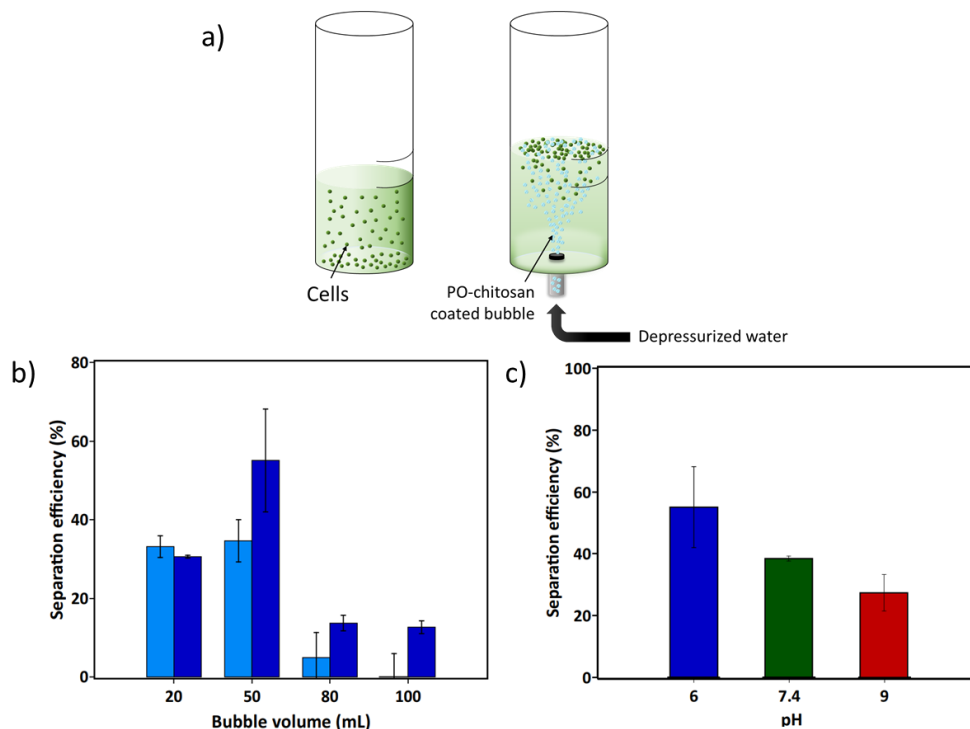
493 **Figure 3: Modulation of the interactions between bubbles and *C. vulgaris* cells by PO-chitosan.** a) Schematic  
 494 representation of PO-chitosan coated bubble and single *C. vulgaris* cell interaction. b) Representative force  
 495 curves obtained for PO-chitosan coated bubble and *C. vulgaris* cell at pH 6. Adhesion force histogram obtained  
 496 for the interactions between PO-chitosan coated bubbles and *C. vulgaris* cell at c) pH 6, d) pH 7.4 and e) pH 9.  
 497 Lighter colors histograms in c, d and e show clean bubble - *C. vulgaris* cell interactions at the corresponding pH  
 498 values.

499 In a next step, to see how these interactions between cells and PO-chitosan bubbles  
 500 influence cell capture and subsequent separation by the bubbles, we performed flotation  
 501 experiments. To produce functionalized bubbles, water containing PO-chitosan was pressurized to 6  
 502 bar for 30 minutes. Then, by introducing the white waters into the flotation beakers, the bubbles and  
 503 surfactants are released into the medium at the same time; since the surfactants are in close  
 504 proximity to the bubbles, they can assemble on their surface. Figure 4a is a schematic representation  
 505 of the flotation process with bubble functionalization, performed in only one step then with no prior  
 506 flocculation. Unless otherwise indicated all the experiments were performed at pH 6. For that, in a  
 507 first set of experiments, 50 mL of PO-chitosan white waters were injected from the pressurization  
 508 tank to each beaker *via* the solenoid valves. Different PO-chitosan concentrations were tested in a  
 509 range from 12.5 to 100 mg/L, and allowed to determine the best conditions, using 25 mg/L of PO-  
 510 chitosan, where the highest separation efficiency was obtained (Supplementary Figure 3). Indeed, at  
 511 low concentrations, for the volume of bubbles used, there is not enough PO-chitosan to coat the  
 512 surface of bubbles resulting in poor flotation efficiency. On the contrary, when higher concentrations  
 513 are used, there is too much PO-chitosan compared to bubbles, thus PO-chitosan molecules may end  
 514 up in the suspension and saturate it, preventing bubbles to interact with cells.

515 To confirm this, we then used the best concentration obtained, 25 mg/mL of PO-chitosan,  
 516 and varied the ratio of bubbles to cells. For that, we decreased or increased the volume of white  
 517 waters injected in the microalgae suspensions; this results in a lower or higher number of bubbles  
 518 and thus in a decreased or increased bubble surface area compared to cells. Four different injected  
 519 white waters volumes were tested (20, 50, 80 and 100 mL); the results obtained are presented in  
 520 Figure 4c. On this graph, the light blue bars correspond to the control conditions (clean bubbles) and  
 521 the dark blue bars correspond to PO-chitosan coated bubbles. The highest separation efficiency was  
 522 of  $55.1 \pm 13.1\%$ , obtained with a white water volume of 50 mL, which is 1.6 times higher than the  
 523 efficiency obtained with clean bubbles ( $34.6 \pm 3.8\%$ ). The fact that using clean bubbles,



524 approximately 30% of the cells could be separated from the culture medium can result from the  
 525 capture of cells by clean bubbles or from a natural flocculation of cells in these conditions followed  
 526 by their capture by bubbles. Lower separation efficiencies, close the ones obtained in control  
 527 conditions with clean bubbles, were found when using both lower volume ( $33.2 \pm 2.8\%$  for 20 mL of  
 528 bubbles) and higher volumes of bubbles ( $13.7 \pm 1.9\%$  and  $12.6 \pm 1.6\%$  respectively for 80 and 100 mL  
 529 of bubbles). The results obtained using 20 mL can be explained by the fact that in this case the  
 530 surface area of the bubbles is not large enough compared to the amount of PO-chitosan available,  
 531 which saturates the suspension and prevents the bubbles from interacting with cells. On the  
 532 contrary, the poor results obtained using larger volumes may be due to too low a concentration of  
 533 PO-chitosan relative to the bubbles, which are therefore not all functionalized with the surfactant,  
 534 resulting in poor interaction with the cells. Furthermore, at these large volumes, the efficiencies  
 535 obtained with clean bubbles also decrease, suggesting that injecting such volumes of bubbles dilutes  
 536 the solution, resulting in a low probability of collision between bubbles and cells. Finally, as we found  
 537 that cells' interactions with PO-chitosan coated bubble are dependent on the pH, we then  
 538 investigated the influence of pH variation of the separation efficiency using 25 mg/L of PO-chitosan  
 539 with an injected volume of white waters of 50 mL. The results presented in Figure 4c show that the  
 540 highest separation efficiency of  $55.1 \pm 13.1\%$  is obtained for pH 6, and decreases gradually to  $38.6 \pm$   
 541  $0.8\%$  at pH 7.4 and to  $27.3 \pm 5.9\%$  at pH 9. This is in line with the FluidFM experiments which showed  
 542 higher interactions at pH 6, with average adhesion values decreasing then at pH 7.4 and pH 9. These  
 543 experiments then prove that flotation efficiency using functionalized bubbles is dependent on the  
 544 interaction that bubbles have with cells; the higher it is, the more efficient the separation process.



545

546 **Figure 4: Flotation experiments of *C. vulgaris* with PO-chitosan coated bubble.** a) Schematic representation of  
 547 one-step flotation experiments. b) Flotation efficiency of *C. vulgaris* with 25 mg/L PO-chitosan coated bubble  
 548 with varying bubble volume at pH 6. Light blue bars correspond to the control condition with clean bubbles, and  
 549 dark blue bars correspond to the test conditions with bubbles coated with PO-chitosan. c) Flotation efficiency of  
 550 *C. vulgaris* with 25 mg/L and 50 mL PO-chitosan coated bubble at varying pH.

551

552 The team of Henderson was the first to use functionalized bubbles to improve microalgae  
553 harvesting by flotation [31,32]. Their first studies on this topic showed that mixing cationic polymers  
554 with water in the saturator of a DAF unit allowed the production of positively-charged bubbles,  
555 which could then interact with negatively-charged microalgae cell surfaces and separate them  
556 without the need for prior flocculation. They then analyzed the effect of different polymers (different  
557 zeta potential and hydrophobic modifications with different groups) on the PosiDAF process and  
558 showed that while a change in the zeta potential had an influence on the interaction between  
559 polymers and bubbles, a change in the hydrophobic moieties incorporated in the different polymers  
560 affected the absorption conformation of polymers on the bubble surface [33]. They then tested these  
561 polymers in DAF experiments and showed that depending on the polymer used, the maximum  
562 removal efficiency stays more or less constant for the same species. However, depending on the  
563 species used, the removal efficiency varies; maximum removal efficiency was around 69% for *C.*  
564 *vulgaris*, while it was of 38% for a first strain of *Microcystis aeruginosa* and 93% for another strain of  
565 *M. aeruginosa* [33]. Then later they made the hypothesis that the separation efficiencies obtained  
566 were dependent on the algal organic matter (AOM) that cell produce, which differ depending on the  
567 species [34]. To test this hypothesis, they removed the AOM from cells and repeated the flotation  
568 experiment with positive bubbles: their results showed a decrease of the separation efficiencies for  
569 all species. Moreover, by substituting the AOM of one strain of *M. aeruginosa* CS-564/01 (the one for  
570 which the highest separation efficiency was obtained) with the second species of *M. aeruginosa*, the  
571 separation efficiency increased to 90%. This thus proved that AOM is indeed an important factor  
572 promoting the attachment of cells to the bubble surfaces [34]. In our case, the interactions between  
573 the PO-chitosan functionalized bubbles and the cells are not based on an electrostatic interaction, as  
574 discussed previously, but rather on a specific interaction between the chitosan backbone of the  
575 molecule, present on the surface of the bubbles, and the polymers on the surface of the cells. Thus,  
576 the very concept of the bubble functionalization strategy is different from the Posi-DAF process, but  
577 similar results could be obtained regarding cell separation. To also test the hypothesis that AOM  
578 could be involved in the interaction with PO-chitosan functionalized bubbles, we also performed the  
579 experiments with cells in stationary phase, under the conditions for which the best separation  
580 efficiency was obtained (pH 6 and 50 mL of injected bubbles). Cells in stationary phase have grown  
581 for a longer period of time (21 days instead of 7 for *C. vulgaris*), and have produced more AOM in the  
582 culture medium. In this case, the separation efficiency obtained was of  $46.1 \pm 9.2\%$ , thus in the same  
583 range as for 7-days old cells. This means that in our case, AOM is most likely not involved in the  
584 interaction, unlike in the case of Posi-DAF and, as discussed earlier, relies on the specific interaction  
585 between chitosan and the cell wall of cells.

586 Another point that needs to be discussed here is the difference between the bubble  
587 functionalization strategy that we develop in this study and another flotation separation process  
588 called foam flotation. Foam flotation is a type of DiAF (Dispersed Air Flotation) where surfactants are  
589 mixed in the suspension to reduce the surface tension of water. Then bubbles are injected, allowing  
590 to create a stable foam where hydrophobic particles are adsorbed [35]. This process, which  
591 originates from the mineral industry [36], is also used for industrial waste water treatment [37] or  
592 plastic recycling [38]. For microalgae harvesting applications, foam flotation uses cationic surfactants  
593 (most often chemical surfactants) that not only stabilize the foam in the system but also enhance  
594 microalgae hydrophobicity, which is generally weak [39]. In both cases, cationic surfactants attach  
595 either to bubbles or cells which are both negatively-charged through electrostatic interactions,  
596 making the bubble surface positively-charged or the cell surface hydrophobic, allowing the  
597 interaction between the two entities. Thus the process we develop in this study is different from  
598 foam flotation, first because of the bubble generation procedure, different between DAF and DiAF

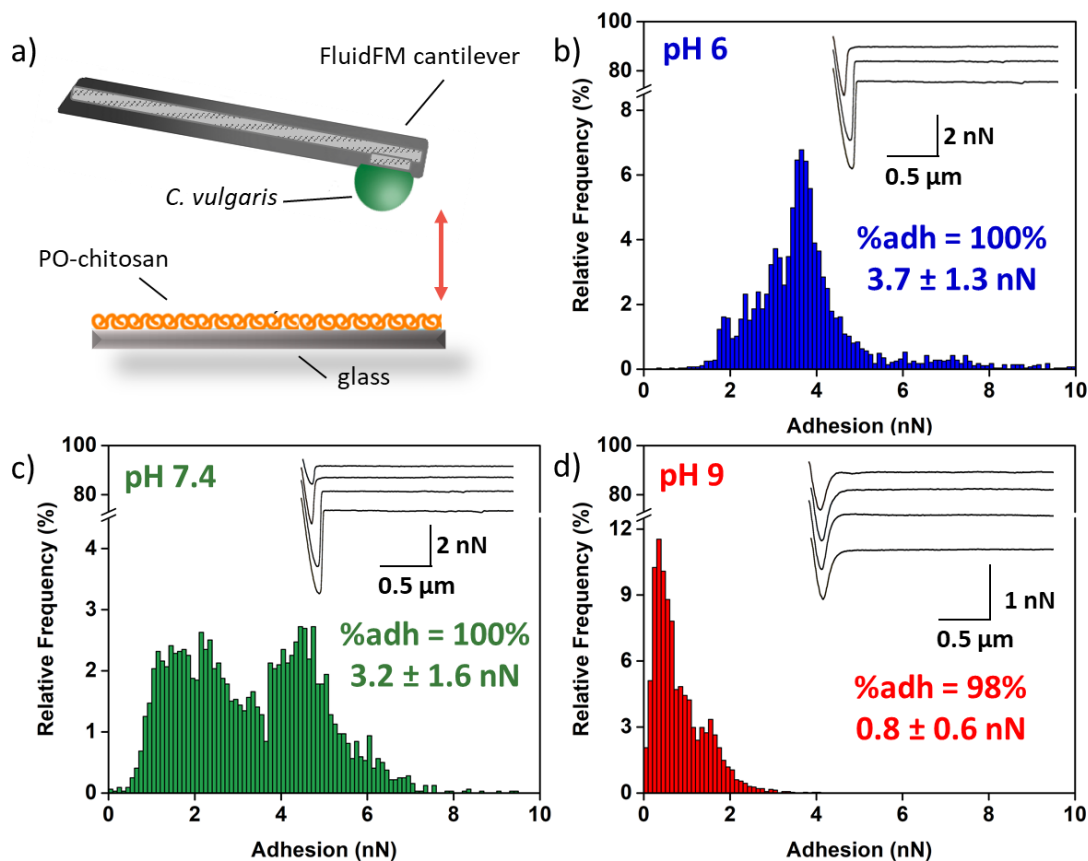
599 [40]. Second because no foam is generated in our process as PO-chitosan is not dispersed into the  
600 medium but mixed with water directly in the pressurization tank to produce functionalized bubbles.  
601 And third because the interaction between functionalized bubbles and cells is based on a specific  
602 interaction between the chitosan backbone at the surface of bubbles and polymers at the surface of  
603 cells and not on an electrostatic one. While the microalgae recovery rates can be higher in foam  
604 flotation than what is obtained here (up to 95% depending on the type of cationic surfactant used),  
605 such processes are usually performed using chemical flocculants (such as CTAB, DAH or DN2) which  
606 contaminate the biomass [41]. Bio-surfactants can also be used, such as rhamnolipid or saponin, but  
607 very few studies have reported on their efficient use for microalgae harvesting in foam flotation  
608 [41,42]. The advantage of the strategy we develop here is that chitosan is a bio-sourced molecule  
609 with no impact on cells, for which the mechanism of interaction with cells is known, making it  
610 possible to optimize the conditions for flotation. But when considering the concept of foam flotation,  
611 a question that can be asked is to know whether PO-chitosan mixed in the suspension could bind to  
612 cells, and act as an effective collector molecule that could enhance the hydrophobic properties of  
613 cells and promote their interactions with bubbles.

614

### 615 **3.4. PO-chitosan is an efficient flocculant for *C. vulgaris* at different pH**

616 To find some answers to this question, we next investigated the interactions of PO-chitosan  
617 directly with cells using AFM. For that, we used FluidFM technology, where single *C. vulgaris* cells  
618 were aspirated at the aperture of FluidFM probes by exerting a negative pressure inside the  
619 microfluidic cantilever, and further used as cell probes to measure the interactions with PO-chitosan  
620 surfaces. This FluidFM method, compared to classic single-cell force spectroscopy methods using  
621 AFM [43], has the advantage of keeping the cells stable on the cantilever even when in contact with a  
622 strongly adhesive surface [17]. In this case also the experiments were performed at different pH (6,  
623 7.4 and 9), given the influence it has on PO-chitosan molecule. The schematic representation of these  
624 experiments is showed in Figure 5a, while the results obtained are presented in Figure 5b-d. At pH 6  
625 (Figure 5b), the retract force curves obtained show a single retract peak happening close to the  
626 contact point, similar to what was observed with bubbles, this time with a smaller average force of  
627  $3.7 \pm 1.3$  nN ( $n= 2851$  force curves with 6 cells coming from 2 independent cultures). As for the  
628 interactions with bubbles, this force signature is typical of non-specific interactions such as  
629 hydrophobic interactions. This first information is important. Indeed, previous results obtained by  
630 performing the same experiments with chitosan surfaces showed force curves with unfolding taking  
631 place far from the contact point, reflecting a specific interaction between chitosan and cell wall  
632 polymers [17]. This means that for PO-chitosan, the interaction is not based on the same mechanism:  
633 instead of a specific interaction with the chitosan backbone of PO-chitosan, it seems here that a  
634 hydrophobic interaction between the hydrophobic octanal groups added to the molecule and the cell  
635 surface is dominant. Thus by changing the molecule, we also changed the physico-chemical nature of  
636 its interactions with cells, and enhanced it as with PO-chitosan the interaction force is 10 times  
637 higher than for chitosan. Similar force curves were obtained at pH 7.4, with an average adhesion  
638 force of  $3.2 \pm 1.6$  nN ( $n= 3194$  force curves with 6 cells coming from 2 independent cultures, Figure  
639 5c). Once we further increase the pH to 9 (Figure 5d), *C. vulgaris* interacts with PO-chitosan through  
640 the same mechanism (force curves present the same single retract peak at the contact point), but  
641 this time with a much lower adhesion force of  $0.8 \pm 0.6$  nN ( $n= 2954$  force curves with 6 cells coming  
642 from 2 independent cultures). Statistical analysis revealed that all differences are significant  
643 (unpaired t-test, p-value of 0.05) meaning that hydrophobic interactions are progressively  
644 suppressed with increasing pH. Now that we know that PO-chitosan interactions with cells are

645 dominantly hydrophobic, these difference observed at different pH can be easily explained. Indeed,  
 646 at pH 9 for instance, both PO-chitosan and cells experience changes in their hydrophobic properties.  
 647 While the WCA of PO-chitosan decreases to 44.6°, the surface of *C. vulgaris* cells becomes hydrophilic  
 648 (no interactions with clean bubbles), and thus interacts less with PO-chitosan. These results are  
 649 important because this means that PO-chitosan could not be used as a collector to enhance the  
 650 hydrophobic properties of cells. Indeed, it interacts dominantly with cells *via* its hydrophobic groups,  
 651 thus the chitosan backbone of the molecule is most likely present on the cell surface, making it  
 652 probably even more hydrophilic. However, given the important adhesion forces obtained especially  
 653 at pH 6 and 7.4, PO-chitosan could perhaps be efficiently used as a flocculant, which could also be an



654 interesting aspect for harvesting.

655 **Figure 5. Interaction between PO-chitosan and single *C. vulgaris* cells at varying pH.** a) Schematic  
 656 representation of *C. vulgaris* and PO-chitosan coated surface interaction with FluidFM. Adhesion force  
 657 histogram between *C. vulgaris* cells and PO-chitosan coated surface at b) pH 6, c) pH 7.4 and d) pH 9. Insets in  
 658 panels show representative force curves obtained

659

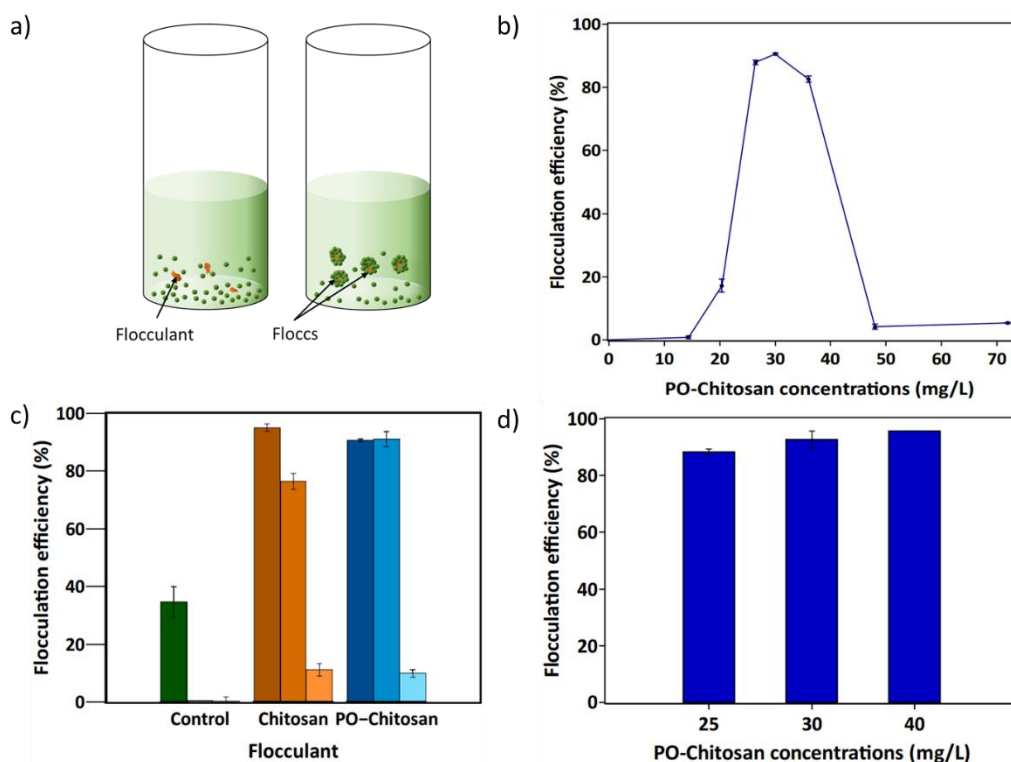
660 To test this hypothesis, we next conducted flocculation experiments with *C. vulgaris* cells at  
 661 different pH with different PO-chitosan concentrations (Figure 6a). In these experiments, no bubbles  
 662 are injected in the solution, cells are mixed with PO-chitosan and then left to settle for 30 minutes.  
 663 The effect of PO-chitosan concentration on flocculation efficiency was first studied at pH 6 where the  
 664 highest flotation efficiencies were reached; the results obtained are presented in Figure 6b. They  
 665 show that for low concentrations of PO-chitosan, until 30 mg/L, flocculation efficiency increases with  
 666 the dose of PO-chitosan used. The maximum flocculation efficiency was of 90.7 ± 0.5%, obtained at a  
 667 concentration of 30 mg/L. However, for concentrations higher than 30 mg/L, flocculation efficiency

668 decreases dramatically and then reaches a plateau at 48 mg/L where the efficiency is close to 5%.  
669 This means that there is a concentration threshold at which the trend is reversed. Such tendency has  
670 already been observed in the case of chitosan, where for small concentrations (up to 10 mg/L)  
671 flocculation efficiency increases with increasing chitosan concentrations whereas for higher chitosan  
672 concentrations (greater than 20 mg/L) flocculation efficiency declines drastically [17,19]. This may be  
673 due to the fact that at high concentrations, the solution is saturated by the large quantity of  
674 molecules present in the microalgal suspension, interfering with their encounter with *C. vulgaris* cells  
675 and probably interacting with themselves rather than with cells. However in the case of chitosan,  
676 only low concentrations (10 mg/L) are needed to achieve high flocculation efficiencies [17]. The fact  
677 that PO-chitosan needs a higher dose to reach nearly 100% of flocculated cells is most probably due  
678 to the fact that PO-chitosan interacts with cells through its hydrophobic groups, which could  
679 substitute only 12% of the amine groups present in chitosan. Thus less groups are available for  
680 interactions, meaning that more molecules are needed to flocculate all cells. For the next  
681 experiments, we then chose to compare results obtained with 10 mg/L of chitosan and with 30 mg/L  
682 of PO-chitosan as these concentrations result in the highest flocculation efficiencies.

683 As for PO-chitosan coated bubbles, we then evaluated the effects of pH variations on  
684 flocculation efficiencies. The results obtained at pH 6, 7.4 and 9 are presented in Figure 6c. At pH 6  
685 (dark bars), both chitosan (orange bars) and PO-chitosan (blue bars) resulted in high flocculation  
686 efficiencies:  $95.2 \pm 1.3\%$  and  $90.7 \pm 0.5\%$ , respectively. While flocculation efficiency decreases at pH  
687 7.4 to  $76.6 \pm 2.4\%$  when using chitosan, it remains constant at  $91.1 \pm 2.6\%$  for PO-chitosan (middle  
688 bars). However, once pH is further increased to 9 (light bars), flocculation efficiencies drop drastically  
689 to  $11.1 \pm 2.2\%$  and to  $10.0 \pm 1.3\%$  for both chitosan and PO-chitosan, respectively. In the case of  
690 chitosan, the situation can be easily explained by the fact that at higher pH, chitosan precipitates and  
691 does not interact with cells anymore. While there, a high flocculation can still be achieved as cells can  
692 get entrapped in the precipitate and flocculated by sweeping, this requires much higher  
693 concentrations of chitosan [16,17]. In the case of PO-chitosan, the situation is different since this  
694 molecule does not precipitate at high pH. But this decrease in flocculation efficiency can be easily  
695 explained by considering the results obtained by AFM, which showed that indeed at pH 9, the  
696 adhesion strength between PO-chitosan and the cells decreases significantly compared to pH 6 and  
697 7.4. Another interesting part of these results concerns the control conditions (Figure 5c, green bars).  
698 Indeed, at pH 6, when no flocculant are used, cells are still able to flocculate with an efficiency of  
699  $33.2 \pm 2.8\%$ . This explains why in flotation experiments using clean bubbles, approximately 30% of  
700 the biomass can be separated. While cells can interact directly with clean bubbles through  
701 hydrophobic interactions (Figure 3c, light blue bars), the fact that they are able to flocculate naturally  
702 at this pH facilitate their collision with bubbles thereby making it possible to float them. However  
703 when the pH is increased to 7.4 and 9, no flocculation at all could be observed, meaning that cells  
704 cannot flocculate anymore naturally. Natural flocculation is often due to the production of EPS by  
705 cells: perhaps at elevated pH, the charge of these EPS changes, as it is the case for microbial EPS [44]  
706 thereby changing their interactions with cells.

707 Thus these results show that indeed PO-chitosan is an efficient flocculant for *C. vulgaris* cells  
708 at pH of 6 and 7.4, and that, as AFM results showed, because PO-chitosan is able to interact through  
709 hydrophobic interactions with cells. However, at these pH values, PO-chitosan molecule could still be  
710 positively charged (pKa of chitosan is of 6.5). Thus to confirm that the flocculation efficiencies  
711 observed at low pH are only due to hydrophobic interactions and not to electrostatic interactions  
712 between PO-chitosan and cells, we performed more experiments. First we measured the zeta  
713 potential of cells at pH 6, 7.4 and 9. The results obtained showed that *C. vulgaris* cells have an  
714 average zeta potential of - 27.1, -26.9 and -26.9 mV respectively. Thus, the global charge of *C.*

715 *vulgaris* cells is negative and does not change depending on the pH. This is a first element, as a  
 716 change in the charge of cells may have explained the decreased flocculation efficiency obtained at pH  
 717 9, although PO-chitosan should not be positively charged at this pH. Second, we repeated the  
 718 flocculation experiments and added 0.2 M of NaCl in the suspension at pH 6 to screen the charges  
 719 present on cells and PO-chitosan molecules. The results obtained are presented at Figure 6d, they  
 720 show similar flocculation efficiencies of  $88.3 \pm 1.1 \%$ ,  $92.7 \pm 2.9 \%$  and  $95.7 \pm 0.1 \%$  using PO-chitosan  
 721 concentrations of 25, 30 and 40 mg/L. These results at the different concentrations are similar to  
 722 what was obtained with no salts added (Figure 6b), showing that indeed, electrostatic interactions  
 723 are not involved at pH 6. Finally we also performed flocculation experiments with stationary phase  
 724 cells at pH 6, which we showed in another study are more hydrophobic than exponential phase cells  
 725 because of the increase of the lipidic fraction in their cell wall upon aging [20]. The results obtained  
 726 showed flocculation efficiencies of  $44.7 \pm 12.6\%$  using chitosan and of  $91.6 \pm 0.9\%$  using PO-chitosan  
 727 (Supplementary Figure 4). In this case the reduced efficiency obtained using chitosan can be  
 728 explained by the fact that the cell wall composition and architecture changes with growth [20]. As  
 729 chitosan interaction with cells is a specific interaction [17], perhaps the polymers with which it  
 730 interacts is less present at the surface of cells, resulting in less interactions and a decreased  
 731 flocculation efficiency. The fact that using PO-chitosan, a similar flocculation efficiency is obtained  
 732 with old cells further confirms that a different mechanisms is involved with this molecule, based, as  
 733 AFM experiments showed, on hydrophobic interactions.



734  
 735 **Figure 6. Flocculation experiments of *C. vulgaris* with PO-chitosan.** a) Schematic representation of flocculation  
 736 experiments. b) Flocculation efficiency of *C. vulgaris* with varying PO-chitosan concentrations. c) Flocculation  
 737 efficiency of *C. vulgaris* with 10 mg/L chitosan and 30 mg/L PO-chitosan with varying pH. Shades of the color  
 738 indicates different pH. Darkest color represent the pH 6, medium color represent pH 7.4 and lightest color  
 739 represent pH 9. d) Flocculation efficiency of *C. vulgaris* obtained with 25, 30 and 40 mg/L of PO-chitosan with  
 740 0.2 M of NaCl at pH 6.

741

742           These results first show that PO-chitosan is also able to efficiently flocculate cells in a pH-  
743 dependent manner, as what was found with functionalized bubbles. Thus the interest of this  
744 molecule is in fact double, and depending on the process, it can be used either to harvest only part of  
745 the biomass using functionalized bubbles and leave cells to continue the culture, or to harvest the  
746 totality of the biomass using flocculation or flocculation/flotation in batch cultures. In flocculation,  
747 while the concentration of PO-chitosan needed is more important than for chitosan, it can however  
748 be used efficiently in more conditions compared to chitosan. First it is efficient at higher pH (7.4),  
749 which is quite important as *C. vulgaris* cultures usually reach pH values close to this in normal culture  
750 conditions. Thus using PO-chitosan, there is no need to first adjust the pH of the microalgae  
751 suspension, saving time and money in harvesting process. Second, it also allows flocculating  
752 stationary phase cells with high efficiency, which is also an important aspect as stationary cells can  
753 yield more of certain products, such as lipids [20]. Finally, another interesting aspect of PO-chitosan  
754 induced flocculation is the size of the flocs produced (see pictures in Supplementary Figure 5). For  
755 instance, using chitosan, cells aggregate into large flocs, that can be too heavy for microbubbles to  
756 carry them to the surface. Therefore using chitosan as a first step in a flocculation/flotation process  
757 may not be very efficient. However, the flocs obtained when using PO-chitosan are much smaller,  
758 probably because of the different flocculation mechanism involved, and can be carried up to the  
759 surface by the bubbles. Finally the originality of PO-chitosan as a flocculant is the fact that it interacts  
760 with cells via hydrophobic interactions. Indeed, most of the used bio-sourced flocculants for  
761 freshwater microalgae harvesting, including chitosan, interact with cells through electrostatic  
762 interactions, and flocculate cells through different mechanisms such as charge neutralization,  
763 bridging and patch mechanisms [13]. Examples of such flocculants are poly  $\gamma$ -glutamic acid ( $\gamma$ -PGA),  
764 a biopolymer produced by *Bacillus subtilis* [45], guar gum [14] or starch, a naturally-occurring  
765 polysaccharide [15]. Because microalgae cells usually have a weak hydrophobicity, most of the  
766 research has focused on these electrostatic interactions and cationic flocculants; hydrophobic  
767 interactions were never explored, as far as we know. But in fact, even if the hydrophobic properties  
768 of cells are weak, the hydrophobic interaction is a strong interaction (typically in the nN range), much  
769 stronger than electrostatic interactions (in the pN range). To give a concrete example of this,  
770 hydrophobic interactions can overcome an electrostatic repulsion between two entities, as we  
771 showed recently when probing the interactions between *C. vulgaris* cells and negatively-charged  
772 microplastic particles [28]. Thus even if the cell surface is slightly hydrophobic, this is enough to  
773 promote a strong interaction with a hydrophobic flocculant, which can result in high flocculation  
774 efficiencies like this is the case with PO-chitosan. In the end, this study, by revealing the potential of  
775 hydrophobic interactions to promote flocculation, shows that microalgae flocculation is not limited  
776 to positively charge biopolymers, and opens-up new avenues for finding new efficient flocculants.

777

#### 778 **4. Conclusions**

779 Because microalgae harvesting is currently the most critical challenge for industry to exploit the full  
780 potential of this biomass, e.g. for biofuel production, new cost-effective solutions are needed. We  
781 propose here a new flotation harvesting process based on the functionalization of bubbles with a  
782 molecule that will improve their interactions with the cells. For this purpose, we based on previous  
783 knowledge on the interactions between chitosan and cells and modified this molecule with  
784 hydrophobic groups to make it amphiphilic. By characterizing this new molecule, we showed that PO-  
785 chitosan could be completely dissolved in water thanks to a low degree of substitution of the amine  
786 functions by the octanal groups of 12%, that indeed the modifications made conferred amphiphilic  
787 properties to the molecule, and that it does not precipitate at high pH unlike chitosan. We then used

788 this molecule to functionalize the surface of bubbles and probe their interactions with cells. As  
789 intended, the functionalization of bubbles allowed increasing in a significant manner their  
790 interactions with cells (from 3.5 to 12.8 nN at pH 6), in a pH-dependent manner. Further flotation  
791 experiments showed that flotation efficiency was directly correlated to the interaction between cells  
792 and functionalized bubbles, as flotation efficiency also changed with the pH. But in our best  
793 optimized conditions (pH of 6, 50% of injected white waters), the removal rate increased from  
794 approximately 30% with clean bubbles to almost 60%, demonstrating the efficiency of this new  
795 flotation process and its potential for continuous microalgae production systems where it could be  
796 used to harvest half of the cells and leave the remaining ones for continuing the culture. Then to see  
797 if PO-chitosan could also be used in different types of harvesting process, we also looked at its  
798 interactions directly with cells, and found that unlike chitosan, PO-chitosan interacts with cells  
799 though hydrophobic interactions, still in a pH-dependent manner. We thus tested its potential as a  
800 flocculant, and found that in fact PO-chitosan is an effective flocculant, able to flocculate nearly 100%  
801 of the cells in the suspension, in more conditions than chitosan, showing the interest of relying on  
802 hydrophobic interactions for flocculation. Here also, the efficiency was pH-dependent, in line with  
803 the results obtained using AFM. Altogether, this study presents an innovative flotation process in  
804 which the functionalization of bubbles with an amphiphilic chitosan allows enhancing cell capture  
805 and separation efficiency. In addition, we show that this molecule can also be used efficiently as a  
806 flocculant, making its interest double for large-scale harvesting applications. In each case, single-  
807 molecule level force spectroscopy experiments allow understanding the nature of the interactions,  
808 providing a complete view of the mechanisms involved and making it possible this way to optimize  
809 their use in large-scale applications.

810

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818

## 819 **Conflicts of interest**

820 The authors declare no conflicts of interest.

821

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