Application of Poly(2-hydroxyethyl methacrylate) Hydrogel Disks for the Immobilization of Three Different Microalgal Species

Short title: Immobilization of microalgae on PHEMA hydrogel disks

Ela Eroglu [a], Risa S. Wijihastuti [b], Elizabeth Grenik [a], Ashiwin Vadiveloo [b], Navid R. Moheimani [b], Xia Lou [a*]*

[a] *Curtin University, Department of Chemical Engineering, Perth, WA 6845, Australia. Emails: ela.eroglu@curtin.edu.au; Tel: +61 8 9266 1075; x.lou@curtin.edu.au; Tel: +61 8 9266 1682*

[b] *Algae R&D Centre, School of Veterinary and Life Sciences, Murdoch University, Murdoch, WA 6150, Australia*

Abstract

BACKGROUND: Algal growth on solid surfaces confers the advantage of combining the algal harvesting and bioprocessing steps at a single stage, in addition to the easier handling of the immobilized cells that occupy reduced amount of space. The current work employed the application of macroporous poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogel disks as a water-insoluble, non-toxic and recyclable immobilization matrix for different microalgal strains (*Nannochloropsis* sp., *Dunaliella salina*, and *Botryococcus braunii*) that offer value-added products for various commercial applications.

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RESULTS: The study demonstrated the effect of variations in the surface characteristics of the algal strains and hydrogel surfaces on the immobilization efficiencies. Gelatin was further used to modify PHEMA hydrogels for achieving higher bioaffinity and surface hydrophilicity. The results showed that highly salt-tolerant microalgal cells (*Dunaliella salina*, *Nannochloropsis* sp.) had significantly higher tendencies to attach on the gelatin-modified PHEMA hydrogel compare to the freshwater *B. braunii* colonies; embedded within an extracellular matrix mainly made of hydrophobic components; which displayed better attachment to the unmodified PHEMA hydrogels.

CONCLUSION: The proposed PHEMA hydrogels are easily-manufactured and highly durable materials with the hydrogel disks still retaining their integrity after several years when in contact with a liquid. PHEMA disks also own the benefits of having adjustable porosities by changing the composition of the polymerization mixture, and modifiable surface properties by simply binding various synthetic or natural molecules on their surfaces, which can bring several new opportunities for harvesting of various microalgal cells with different surface morphologies and chemical compositions.

Keywords: Microalgae; PHEMA hydrogels; Immobilization; Cellular harvesting; Algal biofilm

INTRODUCTION

Immobilization of the cells has various benefits over their free-cell suspensions such as easier handling, conquering smaller space, single-stage cellular harvesting, enhancing the efficiency of wastewater treatment, and permitting the retention and recycling of the algal biomass for high-value-added bioproduct generation at further stages[1-5]. Cells can be immobilized using different techniques including their entrapment within three dimensional gel matrices that can be made of either synthetic or natural polymers, attachment onto the

surface of solid supports, and confinement within liquid-liquid emulsions or semi-permeable membranes; while the two former methods are the most conventional techniques among others [1-3, 6-8]. Challenges to find a proper matrix include surfaces with adequate porosity that would allow the diffusion of the nutrients towards the cells, while permitting the removal of the cellular waste or by-products through their environment [1, 9]. Entrapment of microalgal cells within insoluble materials face some difficulties on the transfer of light and nutrients, which would reflect on lower cell viabilities and slower growth rates compare to their free-cell suspensions [3, 5, 10, 11]. This is mainly due to the slower diffusion rates of the ions and/or light that need to reach the algal cells after passing through the mostly-spherical entrapment material, which typically has a volume to surface ratio larger than thin films[3, 9, 12]. Another important point is to use an insoluble matrix that would keep its integrity without being degraded throughout the process. In the light of those requirements, thin films of macroporous PHEMA and gelatin-modified PHEMA hydrogel were investigated as novel immobilization matrices for the biofilm growth of three different species of green microalgae: (i) *Botryococcus braunii* strain BOT-22, (ii) *Nannochloropsis* sp. (MUR 267), (iii) *Dunaliella salina* (MUR 8). We had chosen to test these three species of microalgae as they are currently targeted for mass production for biofuel generation (*B. braunii* and *Nannochloropsis*) [13-15], high value pigments such as carotenoids, (*D. salina*)[13, 16-18], or high value fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (*Nannochloropsis*) [13, 19]. Biofilm growth on solid surfaces can potentially reduce the overall cost of mass production by reducing the associated costs of dewatering stage, while providing a more efficient harvesting step with the retention of the high-value-added algal biomass for product generations [1, 3, 4].

PHEMA is made of crosslinked polymers of 2-hydroxyethyl methacrylate (HEMA). Due to its swelling properties within water, it is classified as a type of hydrogel material that can sustain its three-dimensional structure [20, 21]. This highly hydrophilic material has various advantages including its stability and inertness at varying environmental conditions such as

temperature and pH; ease of fabrication and alteration of its physical form into any desired shape; and permitting the incorporation of various natural or inorganic molecules into its structure that would change its mechanical/chemical assets and its biocompatibility [20, 22, 23]. Its high biological tolerance permits various applications including vision improvements in the form of intraocular and contact lenses [24, 25]; tissue engineering [23, 26]; dental implants [27, 28]; breast prosthesis [29, 30] or nasal cartilage replacements [31] in plastic surgeries; and controlled drug delivery systems [32-34]. PHEMA hydrogels were also used as an immobilization matrix for various enzymes and biomolecules for enhancing the bioreactor applications[35-37]. Although the utilization of PHEMA for the intraocular and contact lenses mainly involves the use of nonporous and transparent PHEMA hydrogels [24, 25], there is also a significant interest on the fabrication of macroporous PHEMA hydrogels for other biological applications such as the delivery of drugs at higher drug loading capacities [34, 38] and ability to transfer large biomolecules including growth factors and proteins [33]. One of the cost-effective ways to generate macroporous PHEMA hydrogels is the polymerization of HEMA monomers under the presence of free radical initiator to activate a HEMA molecule that will continuously attach onto another one under a chain reaction until the termination of the HEMA supply; a cross linking agent that forms an insoluble network by connecting the PHEMA chains together; and a diluent [38-41]. The concentration of the diluent used during the polymerization process is quite essential for the determination of the porosity of the generated hydrogel. When water is used as the diluent, optically transparent and nonporous hydrogels are produced if the concentration of water in the monomer mixture kept below the critical limits (cited variously in between 40-50 wt%), whereas exceeding those limits would result opaque hydrogels with macroporous morphology as the excess water would induce phase separation during polymerization process [20, 34, 42]. One of the main advantages of the solution polymerization processes is to allow the alteration of the pore sizes and structures by simply changing the concentration ratios of the components within the mixture; i.e. [HEMA]:[solvent] or [initiator]:[crosslinking agent][39]. Applying suitable mixture concentrations at the beginning of

the free radical processes would allow us to produce a stable macroporous material with opaque and spongy characteristics as the presence of hydrophilic groups in their structure let them absorb water [21, 39]. In the current study, gelatin is used for the generation of a modified PHEMA hydrogel with greater surface hydrophilicity and more importantly stronger bioaffinity, which would allow us to compare its bioactivity with unmodified PHEMA hydrogels at different porosities and light transparencies. Gelatin has been known as a surface modifier to PHEMA due to its biocompatibility, low cost and its collagen-based structure [23]. It has been largely used in various biological applications -mostly with mammalian cells- via improving the cellular attachment on culture plates[43, 44], which has also been applied for several microalgal processes such as long-term preservation of microalgae by embedding the cultures on a gelatin based matrix[45] and encapsulation of microalgal oil within a gelatin-gum Arabic complex[46].

MATERIALS AND METHODS

Preparation of PHEMA hydrogels and gelatin-modified PHEMA hydrogels

Previously reported method has been applied to cast the PHEMA hydrogel discs used in this study[27, 34, 38]. In brief, HEMA and water were well mixed in a beaker followed by the addition of the cross-linking agent (ethylene glycol dimethacrylate = EDMA) and the initiators (ammonium persulfate= APS & N,N,N',N'-tetramethylethylene diamine = TEMED), according to the chemical composition listed in **Table S1 and S2**. The solution was then distributed into a 24-well tissue culture plate in a way to obtain 1 ml solution per each well. Polymerization was carried out at room temperature for 3 h, followed by 50 °C for 24 h. Following the polymerization, the discs were removed from the mould and immersed in deionized water for 4 weeks with daily water exchange to remove residual monomers and oligomers. For the synthesis of PHEMA-gelatin disks, 1 wt% gelatin in water was used instead of pure water in

the preparation. Then it followed the same polymerization procedure as stated above for the PHEMA hydrogels. After polymerization, the discs were swollen in 0.5 wt% glutaraldehyde solution at room temperature and 170 rpm (orbital shaker) for 16 hrs to allow crosslinking of gelatin with glutaraldehyde. The discs were rinsed with deionized water for 3 times at 170 rpm for 10 minutes and further purified with daily water exchange for four weeks. It should be noted that all hydrogels can be cast into polymer sheets and cut into any geometry necessary[47]. In this study, the polymer sheets were cut into disks of two different sizes with diameters of 8 mm and 17 mm, having a constant thickness of around 1 mm.

Microalgal strains and culture conditions

Race B *Botryococcus braunii* (BOT-22), *Nannochloropsis sp.* (MUR 267) and *Dunaliella salina* (MUR 8) were used as the microalgal species of this study. *Botryococcus braunii* (BOT-22) was obtained from The Network of Asia Oceania Algal Culture Collections (AOACC), Japan. *Botryococcus braunii* culture was maintained in modified AF-6 medium[48] at a pH around 6.4. The marine Eustigmatophyceae, *Nannochloropsis* sp. (MUR 267) and the Chlorophyceae, *Dunaliella salina* (MUR 8) used in this study were obtained from Murdoch University Algae culture collection. *Nannochloropsis* sp. was grown in F/2 medium with a salinity of 3.5%, while *Dunaliella salina* was grown in F medium with a salinity of 7% as formulated by Guillard (1975)[49]. Both F and F/2 media were made using natural sea water, which was previously collected from the coastal waters off Hillary's Beach, Perth, Western Australia. The seawater was first charcoal filtered and autoclaved prior to the addition of sterile nutrients. Initial algal cell cultures were grown in 250 mL Erlenmeyer flasks, under continuous cool-white fluorescent illumination at incident intensity of around 200 µmol photons $m^{-2}s^{-1}$(PAR).

Initiation and progress of microalgal growth on the surface of PHEMA hydrogels

PHEMA hydrogel disks were initially placed at the centre of a sterile and transparent 6-well

tissue culture plate with an internal diameter of 3.5 cm and a depth of approximately 1.5 cm (Cellstar®). Identified amount of algal culture solutions were slowly added on top of larger (Ø: 17 mm; 0.4 mL) and smaller (Ø: 8 mm; 50 µL) disks (refer to **Figure S1,** Supplementary Information), which were selected according to the near-maximum capacity of the fluid that would stay at the surface of each disk without falling from the sides. Chlorophyll content and quantum yield measurements were both used to validate the uniformity of the initial cell concentrations by inoculating from the algal culture flasks with dark-adapted quantum yields of ~0.65 ± 0.05, which have the initial total-chlorophyll contents as ~1.5 mg/L *D. salina*, ~0.7 mg/L *Nannochloropsis* sp., and ~0.4 mg/L *B.braunii* cells (refer to the Supplementary Information for the calculations of chlorophyll contents). Initial quantum yields of the microalgal cultures were measured after 20 minutes of dark-adaptation period under the room temperature, using a portable fluorometer AquaPen-C (Photon Systems Instruments, Czech Republic). After the first introduction of the cells, the disks were kept on the bench for two days without any additional processing under the illumination of natural cool white fluorescent light with an intensity of 50±5 µmol photon m^{-2} s^{-1} and at a temperature of 25±2 ºC (**Figure S1,** Supplementary Information). This phase is followed by the addition of sterile algae-specific growth media (AF-6 [48] medium for *B. braunii*; F medium [49] for *D. salina* and F/2 medium for *Nannochloropsis* sp.) by slowly dripping from the side of the culture-well (4 mL for larger disks, 500 µL for smaller disks). Then the disks were measured for their dark-adapted photosynthetic activity for every three days. At the end of the growth experiments (with a total duration of 15 days), culture solutions were discarded from the containers followed by the addition of ~3 mL ethanol (70% v/v) by vigorously spraying on the surface of all disks. 3 mL of sterile deionized water were then added on top of the ethanol solution, in a way that the entire disks would be completely immersed within this mixture. These disks, inside the ethanol-water mixture, were kept on the bench for 2 days, followed by the removal of ethanol-water solution and washing with sterile deionized water at least for 3 times. In order to assure that the surfaces of the disks were cleaned from the cells, photosynthetic

activities of the remaining disks were measured. Recycled disks were kept ready within sterile deionized water until their further usage for algal immobilization.

Cell growth of all cultures was carried out on the surface of hydrogel disks under batch conditions at around 25±2 º C and under artificial diurnal-illumination (12 h light / 12 h dark cycle). The light periods of the cycle is provided by natural cool white fluorescent lights at a light intensity of 50±5 µmol photon m^{-2} s^{-1}.

Algal growth measurements

Minimum fluorescence yield

Algal growth was examined by evaluating the photosynthetic activity of the cells through measuring the minimum fluorescence yields (Fo) of the biofilms after their dark-adaptation for 20 minutes before each measurement. Fo values were recorded using a Handy-PEA chlorophyll fluorimeter (Hansatech Instruments, UK) that contains high-intensity LED arrays delivering red-light at a peak-wavelength of 650 nm, while the infrared region of any light source can be blocked by its NIR short-pass filters [50]. Dark-adaptation process allows the re-oxidation of the photosystem-II reaction centre of algal cells, which would lead to the calculation of the minimum fluorescence yield under the lack of any photochemical or non-photochemical quenching of the fluorescence yield[50-52]. Released fluorescence values from the biofilms were later recorded by the integrated software of the fluorimeter, PEA Plus V1.10. All experiments were conducted in triplicates and the standard deviation of each value is given in the form of error bars within the related figure.

Confocal laser scanning microscopy

The viability and thickness of the biofilm were observed under the Confocal Laser Scanning Microscopy (CLSM), Nikon C2+ multispectral laser scanning confocal microscope, which is equipped with 405 nm, 458 nm, 514 nm, 488 nm, 561 nm and 647 nm lasers. The fresh sample of algal biofilm was used for this observation. The surface of each disk was cut into

thinner layers in a way that the light and the laser can easily penetrate the sample. The sample was put upside down on a 35 mm diameter glass bottom dish. The lugol solution was added to *Dunaliella salina* culture for stopping the movement of algal cells prior to the microscopic imaging. No solution addition was necessary for the non-motile species of *Botryococcus braunii* and *Nannochloropsis* sp.. 20x objective was used to capture a three-dimensional biofilm structure and thickness, while Mito Tracker Deep Red laser (640.0 nm) was used for observing the algal cells. Due to their chlorophyll contents, the algal cells autofluorophore in red [53, 54]. The images were recorded and processed by the software package (Nikon Imaging Software (NIS)-Elements) that converted the images into their three-dimensional forms with two constant dimensions (L:632 µm × W:632 µm) and varying biofilm depths (stated as "D" in Figure 5). This observation was done in 2 replicates.

Scanning electron microscopy

Surface analysis of hydrogels, with and without attached algal cells, were investigated by a scanning electron microscopy (SEM, NEON 40EsB) analysis at an accelerating voltage of 3kV on the samples coated with platinum (layer thickness of ~3nm). The hydrogel samples were freeze-dried for a day prior to the SEM analysis. The hydrated hydrogels were previously kept overnight at -40 °C inside a freezer, then the frozen samples were transferred into a vacuum chamber that is connected to a condenser and cooled to -55ºC. For the SEM analysis of the microalgal cultures, given in Figure 2, 200 µL of liquid cultures were initially placed on the surface of the SEM pin stubs with carbon adhesive tabs, and allowed to dry under the laminar hood before being coated with a layer of platinum for the SEM analysis. Size measurements, such as the pore diameters and the sizes of microalgal cells, were calculated with the aid of ImageJ 1.50i software.

RESULTS AND DISCUSSION

Immobilization efficiencies of PHEMA hydrogel disks with different porosities

The first challenge of the present work was to test the immobilization capabilities of two different PHEMA hydrogel disks with different porosities. Opaque E25 PHEMA and transparent E60 PHEMA are the labels of the hydrogel specimens used in this study, where the numbers represent the percentage of HEMA in the polymerization solutions (see **Table S1**) and the capital letter "E" symbolizes the cross-linking agent EDMA. Porosities of PHEMA hydrogels are known to be affected by the variations in the ratios of HEMA to water concentrations present in the polymerization mixtures[20, 27, 34, 39, 42]. **Figure 1** shows the scanning electron microscopic (SEM) images of as-prepared E25 and E60 PHEMA specimens before the introduction of any algal cells. SEM images indicated that the porosity of the disks increased as the percentage of HEMA decreases (or percentage of water increases), making E25 sample more porous than E60 (**Figure 1**). On the other hand, E60 PHEMA specimen's surface was only composed of flakes rather than porous holes (**Figure 1c&d**). The reason to continue testing the E60 sample for algal growth was its more transparent nature compare to E25 PHEMA, which would have facilitated the photosynthesis process by allowing the transfer of the light throughout the entire disk. The diameter of the pores at the surface of E25 PHEMA had a range between 0.6 to 2.5 μm, with an average diameter of around 1.6 μm (**Figure 1a&b**). It should be noted that these pore sizes are calculated according to the SEM imaging of the freeze-dried samples, which might be slightly higher in their actual moist conditions [55]. The porous structure of E25 PHEMA disk makes it a good candidate for serving as a supporting matrix of the microalgal cells, mainly for the *Nannochloropsis* sp. and *D. salina* cells due to their smaller sizes than *B.braunii* cells (**Figure 2**). According to the SEM images of those cells, *Nannochloropsis* sp. and *D. salina* cells have spheroidal shapes with average diameters of around 2.8 μm (**Figure 2a-b**) and 3.5 μm (**Figure 2c-d**), respectively. On the other hand, *B.braunii* cells form large colonies of around

30 μm that are made of pyriform-shaped individual cells with an average length of around 9.5 μm (**Figure 2e&f**). One of the advantages of porous surface structures is the possibility to allow the replication of the cells on their surfaces by facilitating the diffusion of the nutrients towards the cells, while transferring the cellular waste/by-products from the cells through their environment [1, 9]. It is worth noting that porous PHEMA hydrogels produced in the presence of large amounts of water, as reported in this paper, are termed 'phase separation' hydrogel[24]. These materials are well known for their applications as medical implants and tissue engineering scaffolds in which the presence of interconnected pores and the non-toxic nature are essential to facilitate the growth, proliferation and migration of animal cells [24, 25, 39, 56]. Such hydrogels have also been studied for sustained delivery of therapeutic drugs[34, 38] and gas transportation and storage[57]. The feature of open pore channels and its correlation with the transportation characteristics of drugs and gases are extensively studied[58-61].

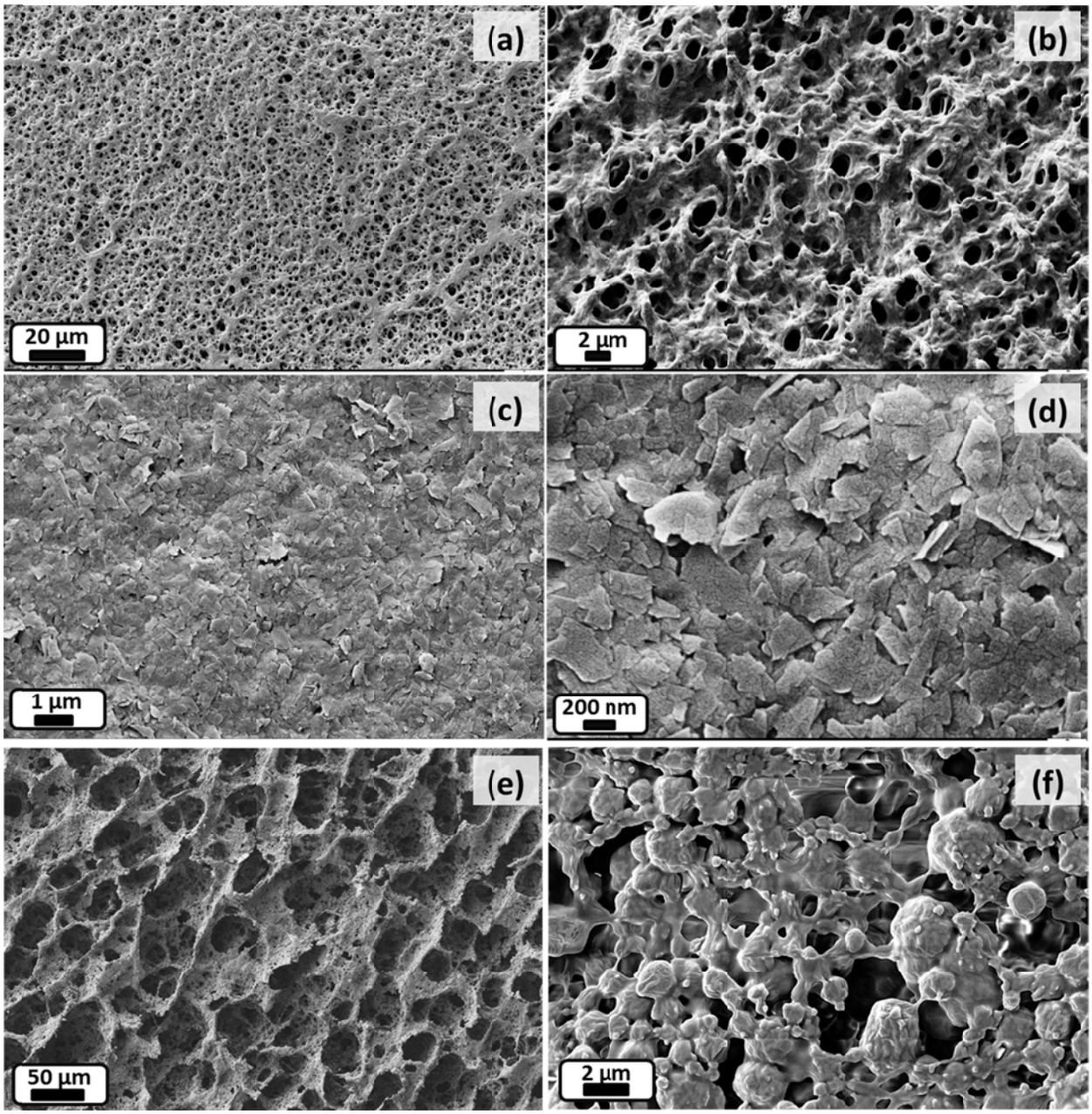

Figure 1. SEM images of as prepared **(a & b)** E25 PHEMA; **(c & d)** E60 PHEMA hydrogels; and **(e & f)** gelatin-modified E20 PHEMA at low and high magnifications. Differences in the magnifications of the variants of hydrogels are due to presenting the best magnification image with detailed surface structure.

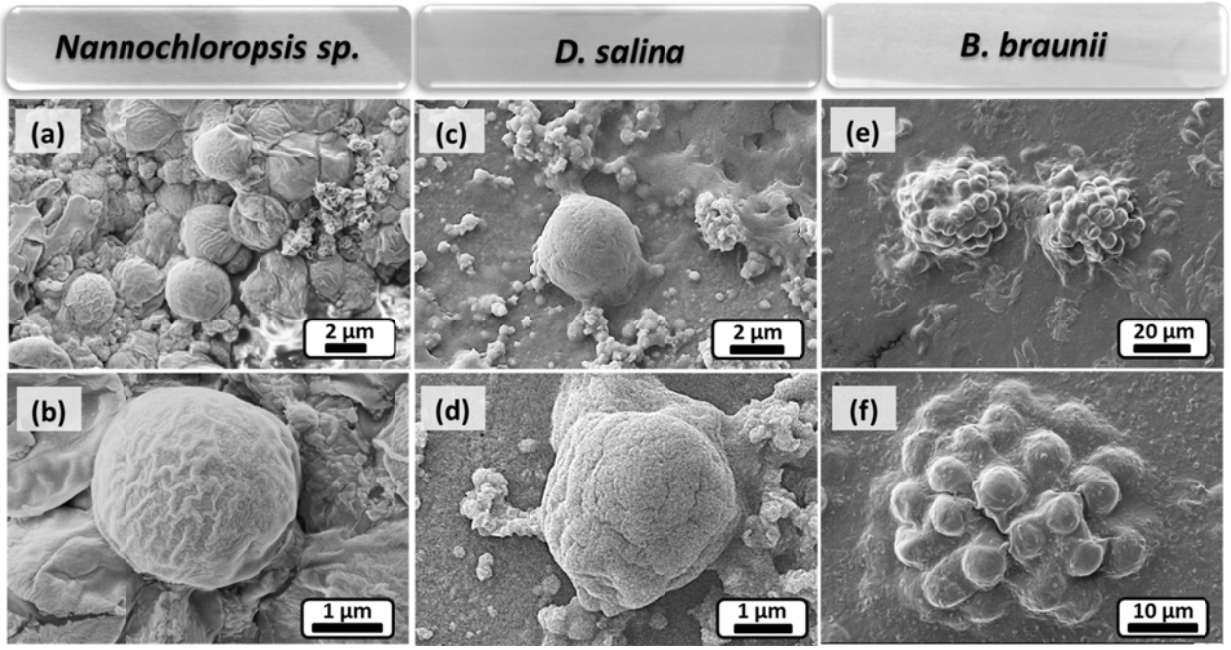

Figure 2. SEM images of air-dried **(a & b)** *Nannochloropsis* sp.; **(c & d)** *D.salina*; **(e&f)** *B.braunii* cell cultures at low and high magnifications.

Immobilization of microalgal strains on the gelatin-modified PHEMA hydrogel and its comparison with the unmodified PHEMA hydrogel disks

The next challenge was to explore the attachment of the microalgal cells on gelatin-modified E20 PHEMA disks. The porosity of the gelatin-modified E20 PHEMA hydrogel disks were also explored under SEM, showing a highly porous structure forming a hallow network – hollow sizes mostly ranged between 10 to 45 µm, with an average diameter of around 25 µm) (**Figure 1e**)-, which has various internal porous passages with smaller pore sizes at a diameter range of 0.5-4.5 µm (**Figure 1f**). The greater pores observed in gelatin modified E20 PHEMA are largely due to the higher amounts of water, in comparison with E25 and E60, used in the polymerization process [27, 34, 40] .

The bioactivity of the hydrogel disks were explored by monitoring the growth of microalgal cells on the surface of their solid supports at different time intervals. Due to the impracticalities of several cellular growth analysis, such as the difficulties observed during cell counting of the colony forming *B.braunii* cells or motile *D.salina* cells, along with the

harshness of the chlorophyll extraction from the surface of the disks; an *in situ* analysis was conducted by measuring the photosynthetic activity of the cells as an indication for the cellular growth without harming either the cells or the disks during the progression of the growth experiment. Minimum fluorescence measurements in the dark-adapted state (F_o) is referred to have a significant positive correlation with the growth of microalgal cells, due to the observed linear relationship between F_o and Chl *a,* allowing us to use F_o results as a non-invasive proxy tool for estimating algal biomass [51, 62, 63]. According to this information, we measured dark-adapted (20 min.) minimum fluorescence values using Handy-PEA chlorophyll fluorimeter (Hansatech Instruments, UK) as an indication for the bioactivity of the tested hydrogels. **Figure 3** shows the variations observed for the photosynthetic activities of three different microalgal cells at different time intervals. The comparative growth tests between E25 and E60 PHEMA specimens indicated the clear success of the more porous E25 PHEMA sample for being a better support for all of the tested microalgal species (**Figure 3a-c**). **Figure 3(d-f)** showed that the gelatin-modified E20 PHEMA hydrogels provided the best support for *Nannochloropsis* sp. and *D.salina* cells (**Figure 3d&e**); whereas unmodified PHEMA disks were more attractive for the cells of *B.braunii* (**Figure 3f**). When gelatin-modified E20 PHEMA was compared with E25 PHEMA specimens at similar dimensions (Ø: 8 mm), *Nannochloropsis* sp. cells had around 20 times higher photosynthetic activity on the surface of the gelatin-modified hydrogel (**Figure 3d**), while this increase was only around 5 folds for *D.salina* cells (**Figure 3e**), where both differences were the highest at the 6th day of the growth experiments. It should be noted that larger E25 PHEMA hydrogel (Ø: 17 mm) had better biofilm activities than its smaller counterpart (Ø: 8 mm) due to the presence of greater area for the cells to form biofilms. Growth of microalgal cells was also observed visually, where the colour changes on the surface of each disk can be seen on the real-time images taken at the beginning, 6th day, and by the last day (15th day) of the growth experiments (**Figure 4**). Note the increasing green colour on the surfaces is due to the increased concentration of algal cells on the hydrogel mats. Larger E25 and E60 PHEMA disks had

their greenest colour by the 6th day due to their coverage with *Nannochloropsis* sp. (**Figure 4N(b&e)**) or *D.salina* cells (**Figure 4D(b&e))**, whereas their gelatin-modified E20 PHEMA equivalents kept their green colour until the 15th day of the experiment with a slight increase on their growth during the second-half of the experiment (**Figures 3d-e**; **4N(i)&D(i)**). It should be noted that the overall duration of the growth experiments for *Nannochloropsis* sp. and *D.salina* cell cultures also lasted for 15 days in order to compare them with *B.braunii* cells with slower growth. Due to the characteristic slow-growth rates of *B.braunii* cells [50, 64], more pronounced delay was observed for reaching the maximal cellular activity as can be seen in **Figures 3c&f** and **4B**. For the case of E25 and E60 PHEMA disks with *B.braunii* cells (**Figure 4B(a-f)**), less area is covered with green colour, which is mostly localized on specific places rather than being spread throughout the hydrogel surface. This might be mainly due to the colony-forming nature of the cells (**Figure 2e&f**) in addition to showing lower photosynthetic activities than *Nannochloropsis* sp. or *D.salina* cells (**Figure 3**). *B.braunii* cells grown on the surface of gelatin-modified E20 PHEMA showed the lowest cellular activities, as also observed with the least colour change during the time course of the growth experiment (**Figure 4B(g-i)**).

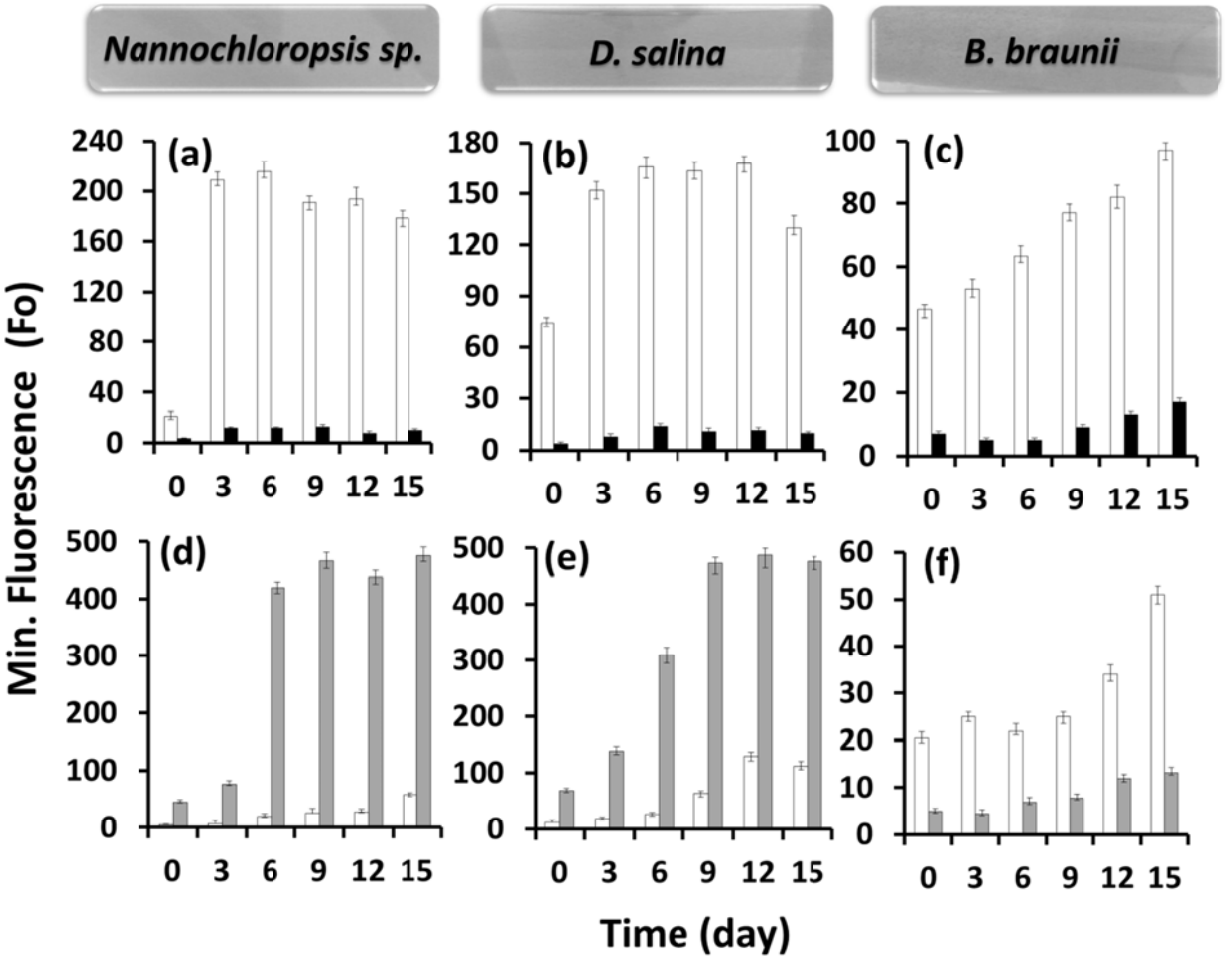

Figure 3. Photosynthetic activities of **(a & d)** *Nannochloropsis* sp.; **(b & e)** *D.salina*; **(c & f)** *B.braunii* cells on the surface of E25 PHEMA (white columns), E60 PHEMA (black columns) and gelatin modified E20 PHEMA (grey columns) hydrogel disks, based on the minimum fluorescence measurements at different time intervals. Note that the upper part **(a-c)** shows the results obtained for the larger disks of E25 and E60 PHEMA having diameters (Ø) of 17 mm; whereas the lower part **(d-f)** belongs to the smaller disks of gelatin-modified E20 PHEMA and E25 PHEMA hydrogel that were initially cast identical (Ø: 8 mm).

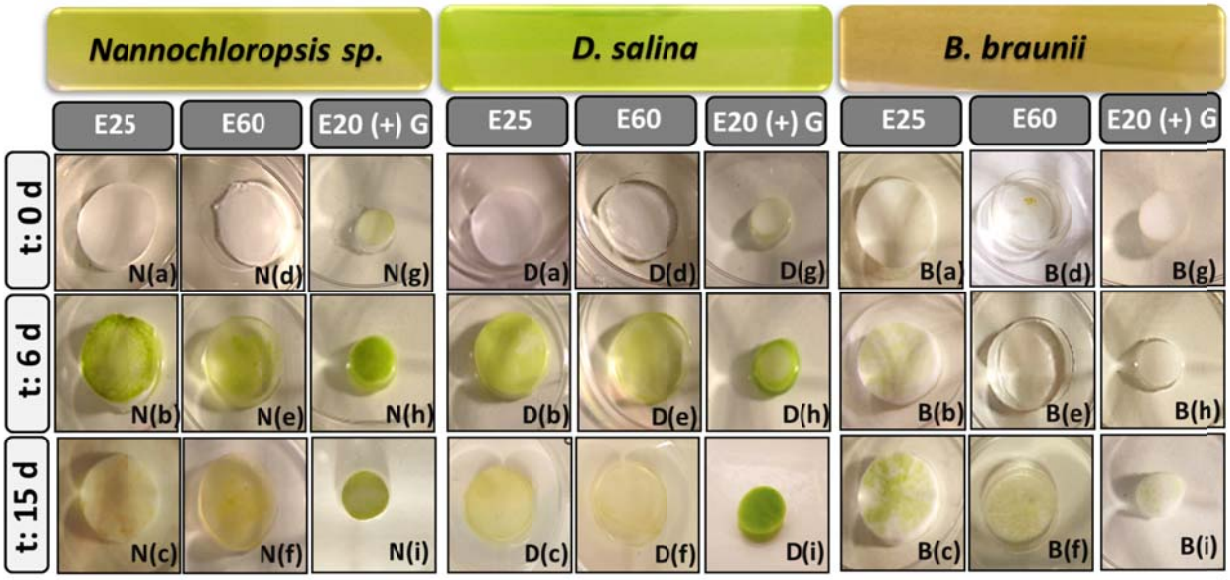

Figure 4. Progress of the algal growth on the surfaces of E25, E60 and gelatin-modified E20 PHEMA hydrogel disks **(1st-row)** at the beginning, **(2nd-row)** after 6 days, and **(3rd-row)** after 15 days from the start of the growth experiments. "N" letter represents *Nannochloropsis* sp.; whereas "D" and "B" letters represent *D.salina* and *B.braunii* cells, respectively.

Surface activity of each disk was also monitored by a confocal laser scanning microscopy (CLSM) using a Nikon C2+ confocal microscope (**Figure 5**), along with the red autoflorescence assets of the algal cells due to their chlorophyll content [53, 54]. The red fluorescence of algal cultures, **Figure 5**, indicates the viability of the cells on hydrogel surfaces, which establishes the nonlethal effect of PHEMA hydrogels on them. It should be noted that the samples were imaged on the 7th day after the initiation of algal growth, representing the approximate half-time of the growth experiment. Gelatin-modified E20 PHEMA had the most concentrated *Nannochloropsis* sp. (**Figure 5c**) and *D.salina* (**Figure 5f**) cells on their surfaces based on the coverage area and the biofilm thickness (D) on each mat. E25 PHEMA hydrogel hosted more *B.braunii* cells on their surfaces (**Figure 5g**), although relatively large biofilm thicknesses of around 40±2 μm were observed on the surfaces of all hydrogel mats covered with *B.braunii* cells (**Figure 5g-i**). Accumulation of thicker *B.braunii* biofilms is due the larger sizes of each individual cells (~10 μm) that also

form large colonies (refer to **Figure 2e&f**), in comparison with the non-colony forming *Nannochloropsis* (**Figure 2a&b**) and *D.salina* (**Figure 2c&d**) cells with smaller cell diameters of around 3-3.5 µm.

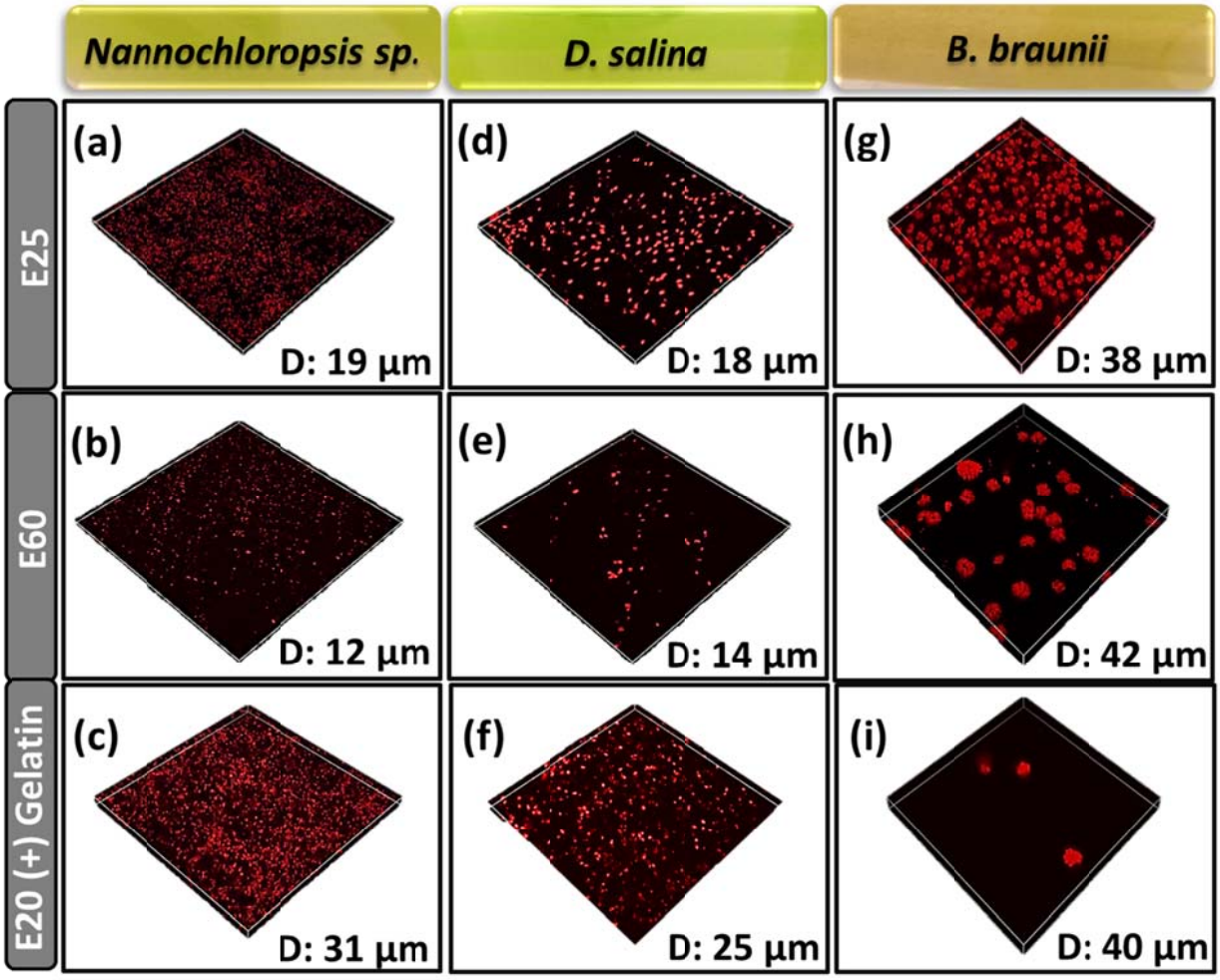

Figure 5. Confocal laser scanning microscopic images of **(a-c)** *Nannochloropsis* sp.; **(d-f)** *D.salina*; **(g-i)** *B.braunii* cells on E25 (first row), E60 (middle row) and gelatin-modified PHEMA hydrogel disks (last row). "D" represents the depth/thickness of the biofilm on a three-dimensional image with constant other dimensions at 632 µm width (W) x 632 µm length (L). Note that the observed red autofluorescence is due to the presence of active chlorophyll pigments in microalgal cells [53, 54].

Combination of the aforementioned results revealed the success of PHEMA disks for the cellular attachment of *N.chloropsis* and *D.salina* cells in a descending order as the gelatin-

modified E20 > E25 PHEMA > E60 PHEMA, whereas this order is different for *B. braunii* cells as E25 PHEMA > E60 PHEMA > gelatin-modified E20 PHEMA. As the two most successful mats, gelatin-modified E20 PHEMA and E25 PHEMA specimens have macroporous structures in common (**Figure 1**), revealing their physical availability to entrap various microorganisms. SEM images of both E25 PHEMA (**Figure 6**) and gelatin-modified E20 PHEMA (**Figure 7**) were also investigated after the immobilization of the cultures, displaying the entrapment of the cells within the porous matrices with the exception of the larger *B.braunii* colonies (**Figure 2e&f**) that are mostly attached on the surfaces of E25 PHEMA hydrogel mats rather than being embedded within its pores, which are relatively smaller than the sizes of the colonies (**Figure 6e&f**). For the case of the gelatin-modified E20 PHEMA hydrogels, spherical morphologies of both *Nannochloropsis* sp. and *D.salina* microalgal cells can be distinguished from their immobilizing hydrogel surfaces by the larger sizes of the algal cells (varying between ~2.5 and ~4 μm; **Figure 2**) than the more distorted spherical droplets of PHEMA polymers (average size ~1.5 μm; **Figure 1**), which is in agreement with the reported observation of freeze-dried E20 PHEMA-only hydrogels by Paterson et al. [55]. Additionally, *Nannochloropsis* sp. and *D.salina* microalgal cells appear to have smoother surfaces under the SEM imaging, which is a typical observation for various algal cells [65].

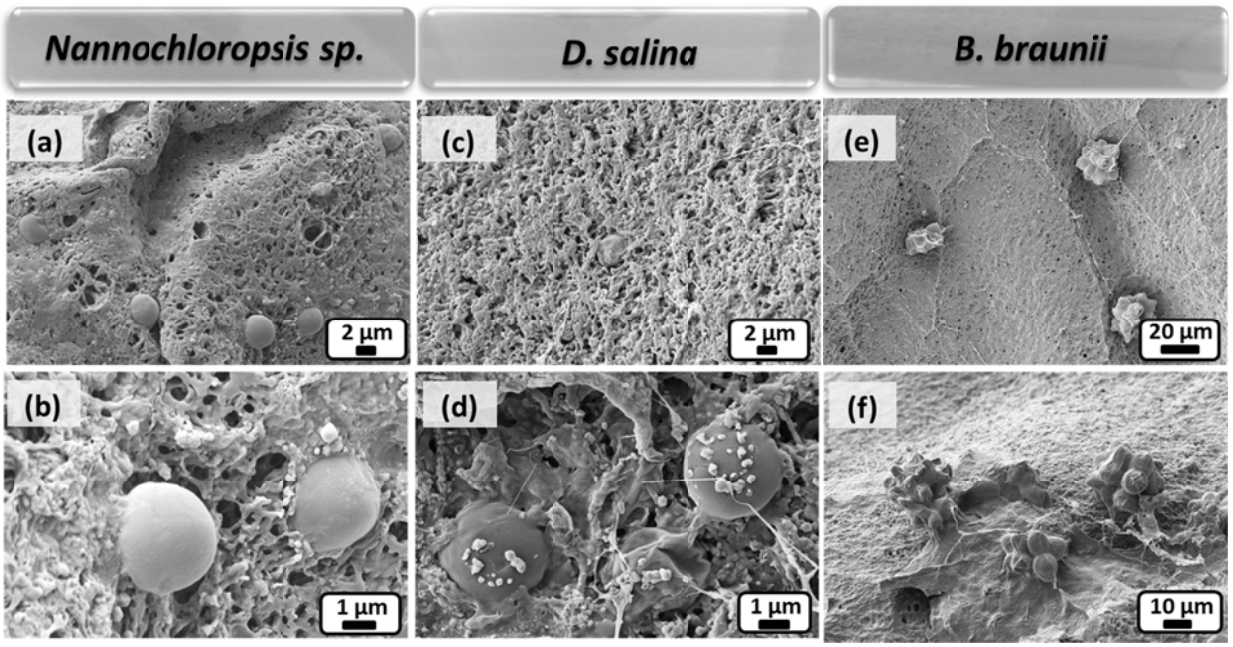

Figure 6. SEM images of immobilized **(a & b)** *Nannochloropsis* sp.; **(c & d)** *D.salina*; **(e & f)** *B.braunii* cells on the surface of E25 PHEMA hydrogel mats at low and high magnifications (samples are taken on the 7th day of the experiment).

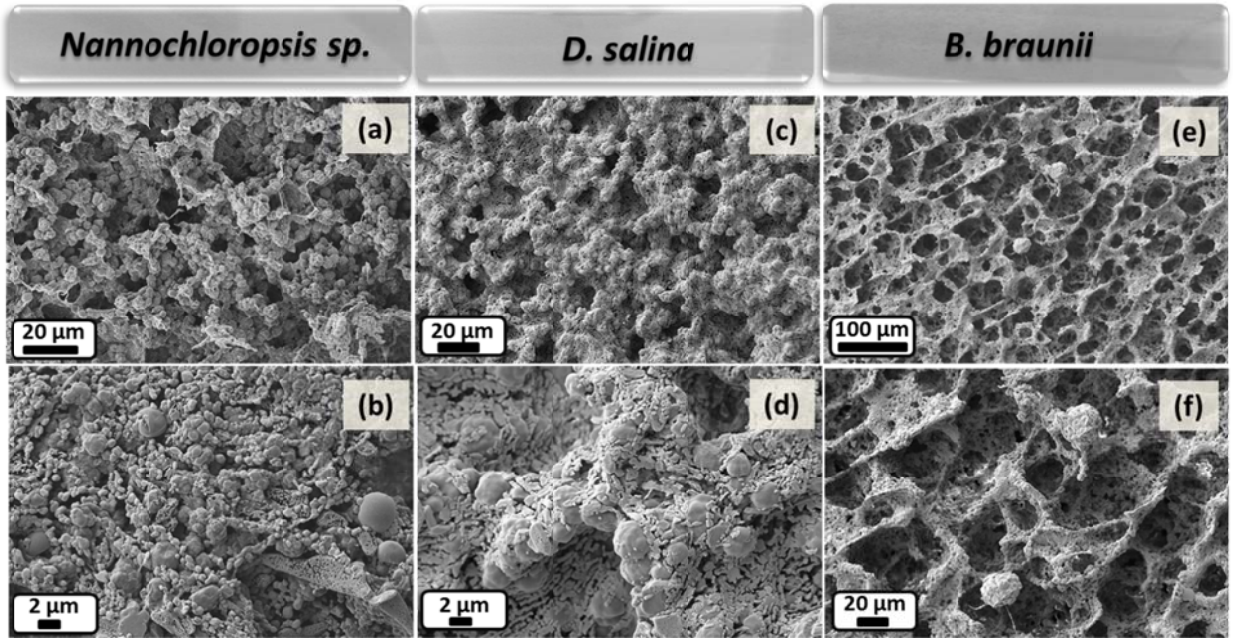

Figure 7. SEM images of immobilized **(a & b)** *Nannochloropsis* sp.; **(c & d)** *D.salina*; **(e & f)** *B.braunii* cells on the surface of gelatin-modified PHEMA hydrogel mats at low and high magnifications (samples are taken on the 7th day of the experiment).

Structural properties of the solid surfaces such as their porosities, sizes of the pores, and surface geometries; morphology of the cells; hydrophobicity and surface charges of both the cells and their solid supports are some of the important parameters that contribute on the efficiency of cellular attachment on to the supporting solid matrix [66]. As two of the unmodified PHEMA hydrogels, the significant differences observed for the bioactivities of macroporous E25 PHEMA and nonporous E60 PHEMA specimens, which showed the importance of the porous surface structures for a proper algal immobilization process. According to the current literature, various porous surfaces had been tested for the immobilization of microalgae, such as electrospun nanofibers of chitosan [9]; polycarbohydrate with grooves [67]; controlled-pore glass [68]; cotton cloth [69]; and cellulose nitrate filter paper [50] revealing the successful entrapment capabilities of porous structures with sufficient pore sizes that can allow the adhesion of the targeted-cells.

Compatible surface hydrophobicity of the solid supports and the cells is an important parameter on defining the mechanism of cellular adhesion to the solid surfaces. The cell wall of an individual *B. braunii* cell is known to have internal fibrillary layer made of mucilaginous polysaccharides and an external-trilaminar-sheath [70, 71], while several individual cells adhere by being embedded within an extracellular matrix composed of oils and various cellular excretes [70]. The hydrophobic nature of the terpenoid substrates present in the extracellular matrix of *B.braunii* microalgal colonies -particularly botryococcene and associated carotenoid hydrocarbons for the B-race *B.braunii* cells [70, 72-75]- might be the main reason for the decreased affinity of those cells on the surfaces of the gelatin-modified E20 PHEMA hydrogels with enhanced hydrophilic properties due to gelatin.

Hydrophilic surface proteins [76] and plasma membrane proteins with extracellular hydrophilic moieties [77] are some of the main mechanisms for the adhesion of *D.salina* cells on solid surfaces. It has been also stated that the salt concentration of the culture media is an important parameter for defining the hydrophilicity of the cell membrane of *Dunaliella* cells, as

the solutions with sodium chloride concentrations lower than 3M (i.e. natural sea water, and the F medium used within the current study [49]) results the domination of the cell membrane with polar groups, indicating increased hydrophilic properties[78]. *D.salina* cells are also known to accumulate extracellular polymeric substances (EPS) on their surfaces with hydrophilic nature -mainly composed of various proteins, polymers, phospholipids, and nucleic acids- that are principally used as a carbon and energy storage material during starvation conditions[79]. These EPSs are the heterogeneous mixture of polyelectrolytes that include some groups such as primary amine, aliphatic alkyl, halide-group, aromatic compounds and polysaccharides [79, 80]; which are compatible with the surfaces of both unmodified PHEMA and the more hydrophilic gelatin-modified PHEMA. The unique motility assets of *D.salina* cells [81] would also increase their cellular migration and replication on the surface of the attachment matrix.

Nannochloropsis sp. cells possess high oil contents[82], like the oil-rich *B.braunii* cells, without forming any colonies. Previous studies revealed that *Nannochloropsis* cell walls have a bilayer structure made of a cellulosic inner layer surrounded by an outer hydrophobic algaenan layer[83]. Despite this hydrophobic algaenan outer layer, presence of negative surface charges might have played a more effective role on the attachment of *Nannochloropsis* sp. cells that clearly showed a higher affinity to the modified PHEMA surfaces with hydrophilic gelatin molecules. The presence of hydrophilic functional groups, such as -OH (hydroxyl), $-NH_2$ (amine), and -COOH (carboxyl), can generate surface charges depending on the pH of the environment[9, 84, 85]. Most of the algal cells have negative zeta potentials, as those aforementioned surface groups create negative surface charges by their deprotonation at higher pH conditions above their isoelectric points [84, 86], which is the case for the salt-water media used for *Nannochloropsis* sp. and *D.salina* cells having a pH value around 8.0 [49]. The isoelectric point of 4.7–5.2 range for gelatin (type B) [87, 88] shows the presence of negative surface charges on gelatin molecules under basic physiological conditions, revealing lesser electrostatic attraction to the negative surface charges of

microalgae. This might show that the amino groups present on the *Nannochloropsis* sp. and *D.salina* surfaces would be one of the main sources of electrostatic interactions with the negative groups of gelatin. When the repulsive energy barrier between two surfaces is low enough, the absorption of negatively charged surfaces onto the surfaces showing zeta potentials of the same signs is still possible, which is more pronounced for the low zeta potentials that are associated with low repulsive energies [89]. It should be noted that the saltwater species (i.e., *Nannochloropsis* sp. and *D.salina*) showed lower absolute zeta potential values than the microalgal species living in freshwater media (i.e. *B.braunii*) [84], which was explained by the high ionic strength of the saltwater medium that would decrease the thickness of the electrical-double-layer formed at the solid-water interfaces by compressing it around the cells [84, 90, 91]. Lower absolute zeta-potential values of salty water species (i.e., around -18.5 mV for *Nannochloropsis* [92]) compare to the freshwater *B.braunii* cells (around -30 mV [84, 93]), would overcome the repulsive energy barrier to show electrostatic attraction between PHEMA hydrogels (around -10 mV[57]) or type-B gelatin molecules (around -12.5 mV [94, 95]) that also have negative zeta potentials at physiological conditions [57, 66, 96, 97]. A comprehensive understanding of the position of attachment-sites, comprising their detailed surface morphology and chemical composition, are still needed to fully endorse the interactions between the cells and their immobilization matrices.

Material recycling experiments also revealed that cleaning the used hydrogel disks with an ethanol/water mixture at the end of each cycle was sufficient to re-immobilize new cells on the surface of the recycled disks while yielding similar bioactivities even after three consecutive cycles (data not shown), providing that the disks were kept within a solvent in between the experiments.

CONCLUSIONS

We have established the use of macroporous PHEMA hydrogel-disks as a water-insoluble and non-toxic support for microalgal growth. Both unmodified PHEMA and gelatin-modified

PHEMA were proven to be highly durable polymer supports, with the disks still retaining their integrity after several years if kept moist within an aqueous solution. Holding the advantage of recycling the disks can contribute to the cost reduction of the overall process. PHEMA hydrogels also have the benefit of adjustable porosity, as it can be simply accustomed by regulating the chemical composition of the polymerization mixture to meet the specific needs of the applications. Attachment efficiencies of PHEMA hydrogels can be simply altered by binding various natural molecules on their surfaces, as also revealed here with their gelatin-modified PHEMA. Algae immobilized PHEMA systems will be further developed by integrating them with waste treatment processes and the generation of various microalgal bioproducts including biodiesel, photopigments and fatty acids.

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