Establishment of primmorphs from three Red Sea sponge species

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Background:
Primmorphs are a special form of 3D-cell aggregates obtained from sponge cells. They can be used as biofermenters for the production of bioactive secondary metabolites. In the commercial development of sponge-derived drug leads, the production of primmorphs is one of the methods proposed to solve the supply problem. In addition, using primmorphs for the production of drugs can preserve the sponge population from extinction by producing enough quantities of the extracts and compounds that present in wild sponges.
Objectives:
The presented work aimed to produce primmorphs of Red Sea sponges *Hemimycale* aff *arabica*, *Stylissa carteri*, and *Crella (Yvesia) spinulata* as long-term cultivation *in vitro* and identify the impact of different cell densities on their formation and growth.
Results:
Microscopic studies suggested that primmorphs are formed through four stages: amorphous large cell floc within 1–3 h; small irregular cell aggregations in 1 day; large primary cell aggregations and round-shaped primmorphs after 3 days. Primmorphs of *C. spinulata* and *S. carteri* remained alive for 3–6 months. The primmorphs of *H. arabica* remained alive for 1 month. Long-term primmorph cultivation *in vitro* allows the creation of a controlled live model under experimental conditions.
Conclusion:
This work may provide a solution to the 'supply problem' in the commercial development of sponge-derived drugs, as primmorphs can be used as biofermenters for bioactive secondary metabolite production. In addition, primmorphs can be used to study the morphogenesis of their sponges at different stages and transdifferentiation as well as the processes of spiculogenesis.

Keywords:
Crella spp, Hemimycale spp, primmorphs, Red Sea sponge, Stylissa sp

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INTRODUCTION

Marine sponges (phylum Porifera) produce the most potent and highly selective bioactive secondary metabolites [1]. As sponges grow comparatively slowly, and their sampling is often difficult, a lot of attention is given to the cultivation of sponges *in vitro* and to the development of their permanent cultures [2,3]. One of the most promising approaches that has been recently proposed is the application of in-vitro culture of sponge primmorphs for the production of bioactive compounds in bioreactors [4].

Multicellular aggregates from a dissociated mixed-cell population of sponges are termed primmorphs [5,6]. A primmorph is an intermediate structure between a single cell and a sponge and can serve as a model for the solution of numerous problems in physiology and cellular and molecular biology [5,7–10]. The primmorphs show a characteristic histology. They are surrounded by an almost complete single cell layer of epithelium composed of pinacocytes [6]. The cells inside the primmorphs are primarily spherulous cells, with a few other cells, mainly amoebocytes and archaeocytes [6]. Electron microscopy revealed that primmorphs are very densely packed sphere-shaped aggregates with a continuous pinacoderm (skin cell layer) covered by a smooth, cuticle-like structure [11]. Viable cultures of sponge primmorphs can be used to produce biologically active compounds that may be of interest to the pharmaceutical industry in the biotechnological production of sponge biomass [11–16].

Primmorphs have been obtained from 25 sponge species, each showing differences in the numbers, sizes, and growth dynamics of aggregates [17]. Some examples are *Dysidea avara*[13], *Ircinia muscarum*[18],

Stylotella agminata, *Hymeniacidon perleve*[4–19], *Xestospongia muta*[20], *Stylissa massa*, *Pseudosuberites* aff *andrewsi*, *Halicondria panicea*, *Haliclona oculata*, *Geodia cydonium*, and *Axinella polypoides*[11], and *Acanthella acuta*, *Hemimycale columella*[16].

The present study aimed at performing long-term cultivation of primmorphs *in vitro* from Red Sea sponges *Hemimycale* aff *arabica*, *Stylissa carteri*, and *Crella (Yvesia) spinulata* and identify the impact of different cell densities on their formation and growth.

We aimed to identify generic conditions that were suitable for the long-term culture of primmorphs. This is important to assist in understanding the in-vitro culture techniques, formation dynamics, and structure of primmorphs. Production of biologically active substances help to solve many problems, particularly related to the treatment of cancer. Use of primmorphs for the production of drugs can preserve the sponge population from extinction by creating enough quantities of extracts and compounds that are present in wild sponges.

MATERIALS AND METHODS

Production of primmorphs

Healthy specimens of Red Sea Demospongiae *C. spinulata*[21] as *Grayella*, *H. arabica*[22], which are found massively encrusted on rocks, and *S. carteri* [23] as *Acanthella,* which is fan-shaped and irregular with sharp ridges, were collected by scuba diving from the site of the Marine Biological Station. The apical parts of young specimens were taken and transported to the laboratory in water bags (Fig. 1).

Sponge specimens were soaked in sterile natural sea water (NSW) supplemented with 25 ppm $CuSO_4$ for 18 h to kill protozoan contaminations and then washed three times with sterile NSW to remove the $CuSO_4$[24]. According to the method of Richelle-Maurer *et al*. [22] with slight modifications, cell suspensions of specimens were aspirated using a sterile syringe.

The effect of inoculum cell density was investigated; cell densities of 10, 30, 50, 70, 80, 100, and 250×10^7 cells/ml were used for both *Crella* spp. and *Stylissa* spp. and cell densities of 10, 20, 30, 40, 50, 60, and 80×10^7 cells/ml were used for *Hemimycale* spp. Cell suspensions with varieties of cell densities were transferred to 75-ml sterile flasks. The flasks were incubated under discontinuous gentle agitation on a rocking plate at room temperature. Cell aggregation and primmorph formation were monitored by means of an inverted binocular microscope and then photographed using a Sony digital camera (Tokyo, Japan). Mature primmorphs were picked up from the flask with a sterile spatula and transferred to separate 25-ml sterile flasks. Each flask contained NSW, which was refreshed every day for long-term culture.

Transmission electron microscope

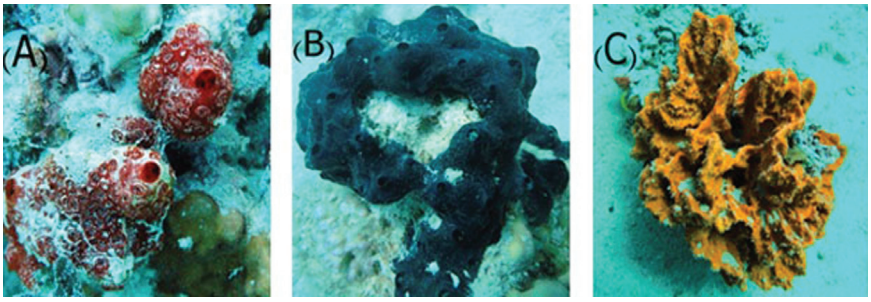
Transmission electron microscopy (TEM) was performed according to the following procedure: fixation of specimens in 3% glutaraldehyde for 1 h, washing in phosphate buffer for 3 h, postfixation in 1% osmium tetroxide (OsO_4) for 1 h, washing in phosphate buffer for 10 min twice, dehydration for 10 min each in 50, 70, 95, and 100% methanol, infiltration in xylene for 10 min twice and then in xylene and resin (2: 1 1 h; 1: 1 1 h; 1: 2 overnight), and, finally, embedding in 100% resin and incubating at 60°C overnight.

RESULTS AND DISCUSSION

Characterizing the dynamics of primmorph formation

A screening of Red Sea sponges was performed to realize a model for the study of fundamental processes

FIGURE 1

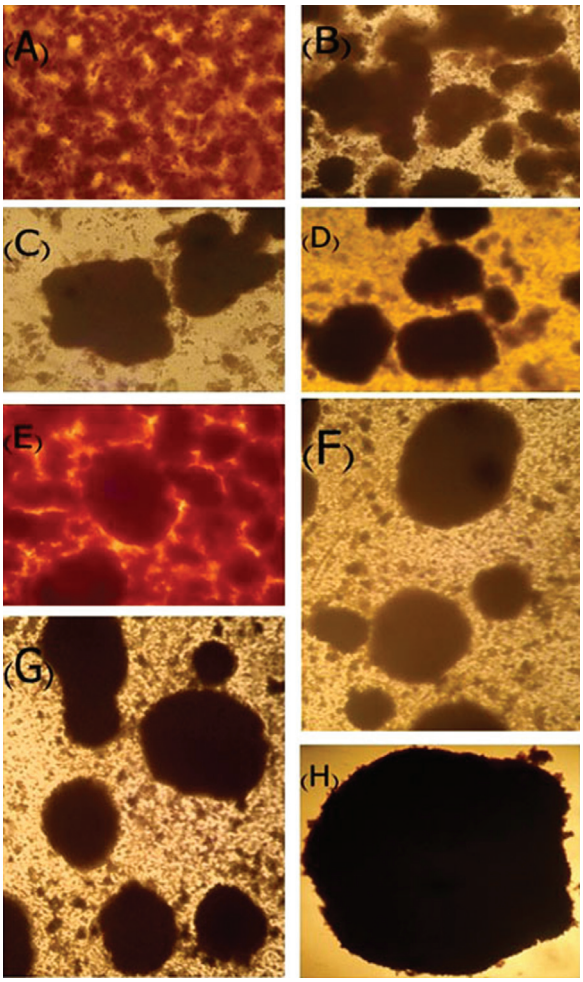


Underwater photos of sponge species (a) *Crella (Yvesia) spinulata*[21] as *Grayella*, (b) *Hemimycale* aff *arabica*[22], (c) *Stylissa carteri*[23] as Acanthella.

in developmental biology and biotechnology, where sponge-cell culture to produce primmorphs might be the most promising method for the production of sufficient sponge biomass for pharmaceutical purposes. We can evaluate the biotechnological potential of the primmorph system when we understand why these aggregates are formed and how they can be used in biotechnological assays. The potency to form these aggregates is likely to be a general characteristic of demosponges [11]. Therefore, in this study, primmorphs were obtained in sterile NSW from three selected Red Sea sponges (class Demospongiae), *C. spinulata*[21] as *Grayella*, *H. arabica*[22], and *S. carteri*[23] as *Acanthella*. The dynamics of the primmorphs' formation process were monitored microscopically using an inverted microscope and usually proceeded as follows: Sponge specimens could be dissociated into cell suspension by means of a physical technique. A cell suspension is termed a mesohyl, which contains heterogenous cells. The primmorph system highlights the importance of cell–cell contacts/communications in successful in-vitro cultivation of sponge cells. It appears that certain cell types are sorted out for primmorph formation from heterogenous cell populations [19]. The interactions among cells as well as between cells and matrix continuously remodel the growth and shape of the adherent aggregates [25]. In the absence of cell–cell contact, cells turn from being telomerase positive to telomerase negative [6–27]. Sponge cells have a high level of telomerase activity, which is a technical indicator of the proliferative ability of cells, when they are present in the state of cell–cell contact [10,27]. Within a few minutes (1–10 min) after dilution of the mesohyl (containing cells that are heterogeneous and free of sponge spicules) with sterile NSW (Fig. 2a), small sponge-cell aggregates were formed (Fig. 2b). Other cell types, which are not included in the process of primmorphs production, were adhered to the bottom of the flask, forming a monolayer.

After a few hours (1–3 h), the small aggregates became visible by eye. They increased in number and size steadily and the amorphous cell flocs reduced with the growth of cell aggregations, making the culture clear. Microscopic observations in case of *C. spinulata* and *H. arabica* revealed that the aggregates became compact with undefined morphology and increased cell density inside (Fig. 2c). In the case of *S. carteri* the aggregates had regular rounded forms representing a defined morphology (Fig. 2d). As aggregation continued, dense cell aggregates transformed to early-stage primmorphs, which were characterized by

Figure 2



Microscopic observation of primmorph formation of *Crella (Yvesia) spinulata*, *Hemimycale* aff *Arabica*, and *Stylissa carteri*. (a) Cell suspension (×150). (b) Cell aggregates 1–10 min after inoculation (×150). (c) Cell aggregates became compact after 3 h (×300). (d) Aggregates had regular rounded forms in *S. carteri* (×300). (e) Early stage of primmorphs 100–400 μm in *C. spinulata*. (f) Early stage of primmorphs 50–300 μm in *H. arabica* and *S. carteri*. (g) Primmorphs after 2 weeks from *H. arabica* and *S. carteri* (300–400 μm). (h) Primmorphs after 3 weeks from *C. spinulata* (500 μm) (×300).

a more or less spherical shape, high density, and a rough surface layer. As the cell–cell contacts in primmorphs are established mostly during the first hours of culture, sterility was vital during the first hours of the culture. Pomponi and Willoughby [28] reported that cultures that are not sterile or in which antibiotics have not been added will become contaminated with proliferating bacteria and protozoa within 1–3 days. Microbial and protozoan contamination as well as poor cell growth prevents successful culture [29].

Cell culture was continued with nonsterile NSW, with daily changes. The use of nonsterile NSW rather than sterile NSW had no influence on the production of primmorphs [30]. Moreover, the NSW was not

Table 1 Primmorph formation from three Red Sea sponge species Crella (Yvesia) spinulata, Hemimycale aff Arabica, and Stylissa carteri

Species	Early stage		Mature		Long-term	
	Size	Cultivation time	Size	Cultivation time	Size	Cultivation time
Crella (Yvesia) spinulata	0.1–0.4	3	0.4–0.5	21	2–2.5	180
Hemimycale aff *arabica*	0.05–0.3	3	0.3–0.4	14	1–1.2	30
Stylissa carteri	0.05–0.3	3	0.3–0.4	14	1.5–2	90

supplemented with any nutrients; that is, the production and maintenance of primmorphs took place under nutrition obtainable naturally in NSW and cells of sponges as the specific symbiotic microbial flora provided the cells with necessary nutrition. Further, unnatural conditions for the development of dissociated cells of sponges can be considered as adverse ecological factors.

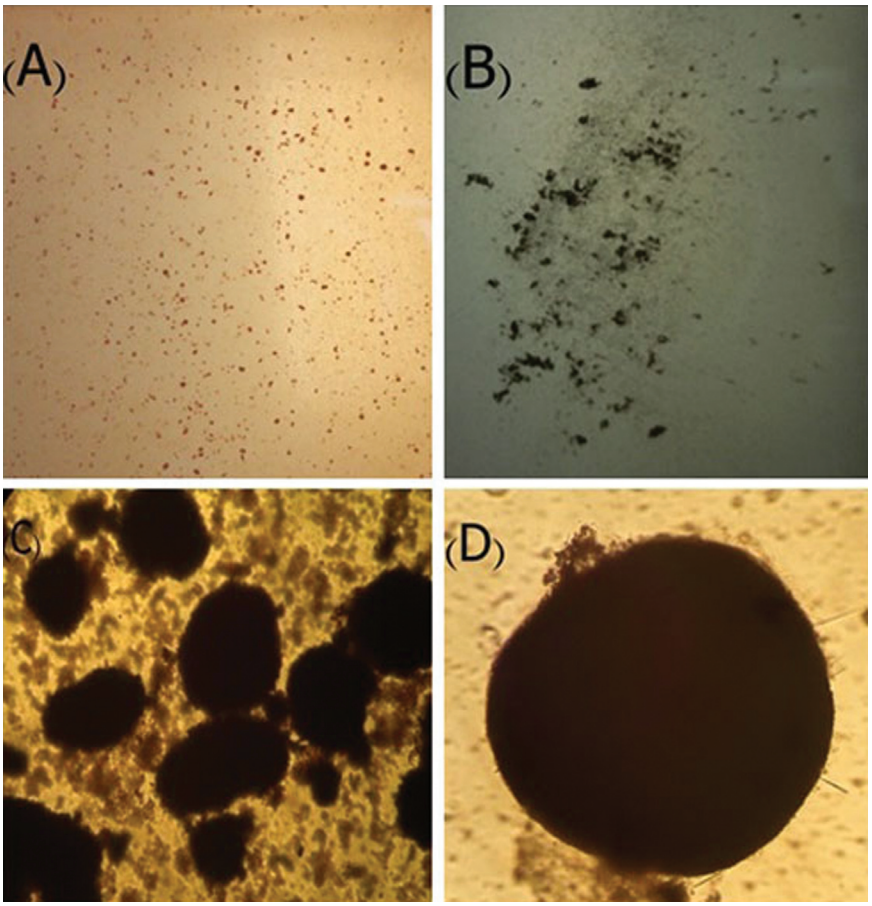
Primmorphs of the three species had different sizes and formation times (Table 1). In 3 days, early-stage primmorphs were formed in sizes ranging from 100 to 400 µm in the case of *C. spinulata* and from 50 to 300 µm in the case of *H. arabica* and *S. carteri*. Subsequently, over 3 weeks primmorphs of *C. spinulata* grew to be rounded, elongated, and surrounded with a smooth 'skin layer' with a diameter between 400 and 500 µm. A shorter period of 2 weeks was required for the formation of rounded primmorphs of *H. arabica* and *S. carteri*, with a diameter between 300 and 400 µm. The difference in primmorph sizes between species might suggest that the size of primmorphs is sponge-species dependent [4].

After development of the smooth skin layer, primmorphs were almost perfect spheres. The light microscope picture showed that primmorphs possess a coating layer, the pinacoderm. The formation of a pinacoderm represents the first step in the reorganization of tissue-like structures. This stage represents the end of the aggregation of cellular material and separation of the internal milieu from the external environment by a continuous pinacoderm [5]. In this respect, it is impossible to overlook the resemblance of primmorphs (smooth spherical aggregates covered with a collagen-like skin layer) with the natural resting-stage gemmule. Gemmule is provided with a pinacoderm that separates the internal cell mass from the environment [5,31]. The cells that ultimately constitute fully formed mature gemmule are referred to as thesocytes and are, in fact, resting archaeocytes [32].

The major objective of the present study was the establishment of suitable conditions that would support a long-term sponge Primmorph culture *in vitro* (Fig. 3a and b). Consequently, primmorphs of *C. spinulata* and *S. carteri* could be kept for 3 months with continuous NSW changes. However, most of the primmorphs lost their smooth skin and started to disintegrate, especially those from *S. carteri*, whereas the primmorphs of the species *C. spinulata* were more tolerant for 6 months. The primmorphs of *H. arabica* could be kept for 1 month, after which most of the primmorphs lost their smooth skin and started to disintegrate. Relative to others, primmorphs of *Suberites domuncula* were kept in culture in the seawater/antibiotics medium for over 5 months in viable state [5,6]. Sipkema *et al.*[11] observed that primmorphs of *S. domuncula* lost their smooth skin and started to disintegrate between 4 and 5 months. The longest period was recorded for primmorphs of the freshwater sponge *Lubomirskia baikalensis*, where the primmorphs continued viable for more than 10 months [10]. Noticeably, a partially unsolved problem in long-term sponge cell culture is the contamination by protozoans and bacteria. Such contamination shortens the culture duration and thus prevents a continuous cell line from being maintained [33].

Microscopic analysis of the mesohyl revealed that no spicules presented in the cell suspension and during the early stages of primmorph formation. During 2 weeks of the primmorph formation process, spicules were observed dispersed in the medium of the formation process. During the long-term primmorph maintenance, spicules (silica-based skeletal elements) were observed protruding from the thin rim region that surrounds the body of the primmorphs (Fig. 3d). It is interesting to note that the spicule formation demonstrated in primmorphs can be considered an early step in morphogenesis [11]. The natural Red Sea water has enough silicate (Na_2SiO_3) (1.33 µmol/l) [34] for siliceous Red Sea sponges to build their spicules skeletons. Therefore, concentration of silicate influenced the formation and growth of spicules in primmorphs of *C. spinulata*, which were cultured and kept in natural Red Sea water. TEM analysis showed the formation of spicule (monactinal spicule) in primmorph as an axial filament that was characterized by rods filled with highly dense

Figure 3



Mature primmorphs seen with the naked eye. (a) Long-term maintenance of primmorphs from *Crella (Yvesia) spinulata* for 6 months (2–2.5 mm) (×2). (b) Long-term maintenance of primmorphs from *Hemimycale* aff *arabica* for 1 month (1–1.2 mm) (×2). (c) Long-term maintenance of primmorphs from *Stylissa carteri* for 3 months (1.5–2 mm) (×300). (d) Spicules protruding from the thin rim region that surrounds the body of the primmorph (2.5 mm) (×300).

material. In Demospongiae, initiation of spicule formation starts intracellularly within specialized cells called sclerocytes. There an axial filament is assembled in organelles around which the first siliceous deposits are layered [35]. Spicules are extruded from the cells into the mesohyl, where their final sizes and shapes are completed. Thickening of spicules proceeds by apposition of concentric silica layers [35,36].

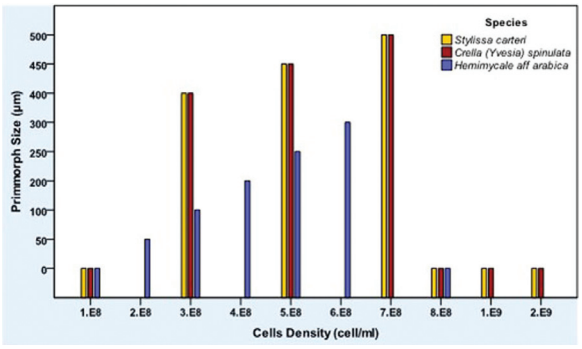
It should be mentioned that the growth of spicules is a fast process [37,38]. However, the process of spicules formation does not occur in some species, as we found in *H. arabica* and *S. carteri*. This may be overlooked or can be initiated with the stimulation of exogenous silicate. As mentioned above, we indicated that spicules formed in primmorphs during in-vitro cultivation in NSW with no addition of exogenous silicate. However, Le Pennec *et al.* [39] reported that when primmorphs were incubated in the absence of exogenous silicate for 5 days no spicules could be seen by microscopic inspection. However, when primmorphs were incubated in the presence of 250 µ mol/l exogenous silicate, bundles of spicules were found. Therefore, optimum concentrations of exogenous silicate may be required to stimulate spicule formation in some sponge species.

Effect of inoculum cell density

The primmorph formation process and sizes depend on the cell densities in the diluted mesohyl as well as on the sponge species (Fig. 4). In case of *C. spinulata* and *S. carteri*, a cell density of 10×10^7 cells/ml led to a reduction in cell adhesion and did not exhibit any development of cell aggregations or primary primmorphs. Cells densities of 30, 50, and 70×10^7 cells/ml led to morphogenesis of cell aggregations that could develop into primmorphs with diameters of 400, 450, and 500 µm, respectively, whereas cells densities of 80, 100, and 250×10^7 cells/ml notably increased the size of adherent mesh aggregations that did not exhibit any development of mature primmorphs.

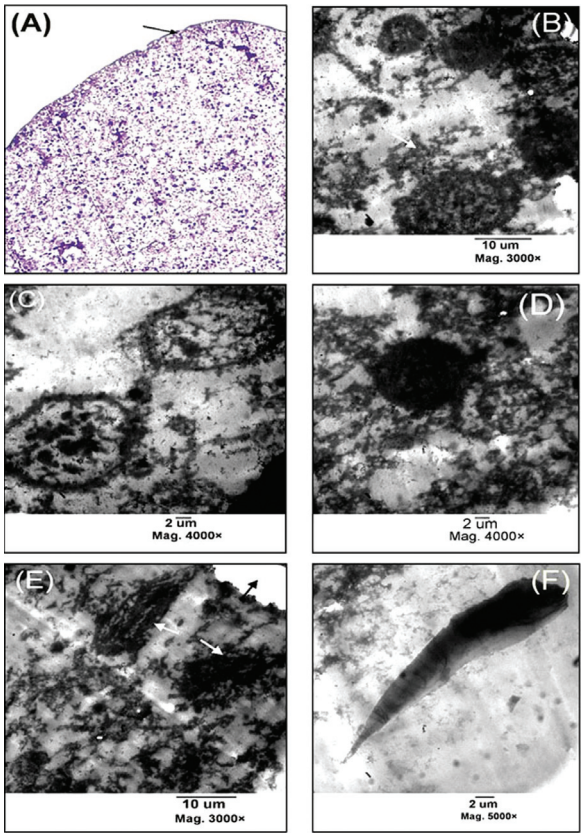
In the case of *Hemimycale* spp. cells, a density of 10×10^7 cells/ml led to a reduction in the cell

Figure 4



Effect of cell densities on the primmorph formation process.

Figure 5



Transmission electron microscopy (TEM) of primmorphs. (a) Light microscopy TS of primmorphs (black arrow; single cellular layer of pinacocytes). (b) Spherule cells (white arrow; collagen-rich mesohyl). (c) Amoeboid archaeocytes with nucleolated nucleus. (d) Archaeocytes with endoplasm loaded with dense irregular granules showing dark color. (e) Choanocytes near the canal-like structure (black arrow; canal – white arrow; flattened, fusiform cells). (f) Formation of spicules in primmorphs.

adhesion mechanism and did not exhibit any development of aggregations or primary primmorphs, whereas cell densities of 20, 30, 40, 50, and 60×10^7 cells/ml led to morphogenesis of cell aggregations that developed into primmorphs with diameters of 50, 100, 200, 250, and 300 µm, respectively. Moreover, microbial contamination was observed in cell density of 80×10^7 cells/ml, which led to cell death.

Transmission electron microscope

Cross-sections of primmorphs under a light microscope showed a single cellular layer of epithelial-like cells termed pinacocytes, surrounding the internal part, which is composed of spherules cells (Fig. 5a). TEM of primmorphs was performed, which revealed that it was packed primarily with spherule cells of different sizes that were densely surrounded by intercellular collagen-rich mesohyl (Fig. 5b). Archaeocytes constitute the major cell fraction in primmorphs; these have the largest size (8–10 µm) and most variable shapes (amoeboid, granular, and globular). Their morphological features show a rough endoplasmic reticulum, nuleolated nucleus, and endoplasm loaded with dense irregular dark-colored granules (Fig. 5c and d). Choanocytes are flattened, fusiform cells measuring 5–6 µm and were observed near the canal-like structure, with their pointed end opening into the canal-like structure (Fig. 5e). One of the major events in the morphogenesis of primmorphs at different stages and transdifferentiation of their cells is the formation of canal-like structures. Müller *et al.*[13] observed canal formation when primmorphs were cultivated in an aquarium for 3 weeks. Hence, Sipkema *et al.*[11] hypothesized that primmorphs have the capacity to develop into functional sponges. Perović-Ottstadt *et al.*[38] described choanocytes, which are the motor cells that drive the water through the aqueous canal system. In the case of the three selected species, TEM analysis showed choanocytes, which are flattened, fusiform cells attached to a canal-like structure (choanocyte chamber or water canal) with their pointed end, and the formation of spicules (monactinal spicules) in primmorphs, which was observed as an axial filament characterized by a rod filled with highly dense material (Fig. 5f).

CONCLUSION

The present study achieved the production of primmorphs from Red Sea sponges *H. arabica*, *S. carteri*, and *C. spinulata* as long-term cultivation *in vitro* and identified the impact of different cell densities on their formation and growth. This work may solve the 'supply problem' in the commercial development of sponge-derived drugs, as primmorphs can be used as biofermenters for the production of bioactive secondary metabolites. In addition, primmorphs can be used to study the

morphogenesis of sponges at different stages and transdifferentiation as well as the processes of spiculogenesis.

Conflicts of interest

There are no conflicts of interest.

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