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Cyanobacterial extracellular polymeric substances (EPS): Production and antitumor activity evaluation

Beatriz da Silva Cruz

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Dissertação de Mestrado em Biologia Celular e Molecular
apresentada à Faculdade de Ciências da Universidade do Porto

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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Abstract

Cancer therapy is still hampered by the negative side effects of chemotherapy and radiotherapy, and there is an urgent need to discover new therapeutics, namely cost-effective antitumor agents from natural sources. The cyanobacterial extracellular polymeric substances (EPS) are mainly composed by heteropolysaccharides that can remain attached to the cell surface or be released into the environment (RPS). Their unique features have shown great potential for a wide range of biotechnological and biomedical applications. However, the studies about their action as antitumor agents are still very limited. Previously, it was reported a strong antitumor activity regarding the RPS from a *Synechocystis* mutant on a Group 3 sigma factor (Δ*sigF)* towards human melanoma (Mewo), thyroid (8505C), and ovary carcinoma (A2780) cell lines. Furthermore, this carbohydrate polymer was extensively characterized, and since it revealed high peptide (~27% w/w) and sulfate (≈12% w/w) contents, the impact of these features in its antitumor activity were evaluated in this work. For this purpose, and in an attempt to optimize RPS production, the *Synechocystis* Δ*sigF* cultures were grown in bioreactors. Subsequently, the polymer was isolated and its peptide and sulfate content were manipulated. The polymer variants obtained were tested *in vitro*, towards the human melanoma (Mewo) cell line, and *in vivo*, by performing the chick embryo chorioallantoic membrane (CAM) assay. Our results showed that aeration increased the growth rate of *Synechocystis* Δ*sigF* cultures but not the amount of RPS produced per cell. However, the polymer yield obtained after 30 days of culture in aerated bioreactors was 1.5-fold higher than in Erlenmeyer flask, indicating that this is a good system to increase Δ*sigF* polymer production. Previously, it was also shown that the Δ*sigF* polymer with reduced peptide content (reduction of ~40%) has stronger antitumor activity *in vitro* towards the Mewo cell line, compared to the unmodified Δ*sigF* polymer. Here, the half maximal inhibitory concentration (IC_{50}) for the polymer variant with reduced peptide content was determined, and the antitumor activity of both variants was validated *in vivo* using the CAM model. Moreover, the Fourier transformed infrared spectroscopy (FTIR) allowed an insight into the functional groups that can be related to the polymer's bioactivity.

In summary, this type of cyanobacterial polymers can be a promising platform for the development of a bioproduct suitable for tumor treatment, namely for melanoma.

Keywords: cyanobacteria; extracellular polymeric substances (EPS); Δ*sigF* polymer; polymer variants; antitumor activity (*in vitro*; *in vivo* CAM model).

Resumo

Os atuais tratamentos contra o cancro apresentam diversas contraindicações devido aos efeitos secundários característicos da quimioterapia e radioterapia. Isto tem levado a uma extensa procura de novas estratégias terapêuticas, como, por exemplo, o uso de agentes antitumorais de origem natural com elevado custo-benefício. As substâncias poliméricas extracelulares (EPS) produzidas pelas cianobactérias são maioritariamente constituídas por heteropolissacarídeos que podem permanecer ligados à superfície celular ou serem libertados para o meio (RPS - *released polysaccharides*). Devido às suas características únicas, os EPS apresentam um grande potencial para uma vasta gama de aplicações biotecnológicas e biomédicas. No entanto, os estudos relacionados com a ação destes polímeros como agentes antitumorais são ainda bastante limitados. Recentemente, foi demonstrado que RPS produzidos por um mutante de *Synechocystis* num fator sigma do Grupo 3 (Δ*sigF)* apresentam uma forte atividade antitumoral contra linhas celulares de melanoma (Mewo), cancro da tiroide (8505C) e carcinoma de ovário (A2780). Para além disso, o polímero do Δ*sigF* foi extensivamente caracterizado, tendo sido revelada a presença de grande quantidade de péptidos (~27% p/p) e grupos sulfatos (~12% p/p). Assim, neste trabalho, foi avaliado o possível impacto destas características na atividade antitumoral do polímero Δ*sigF*. Inicialmente, e de forma a otimizar a produção de RPS, culturas de *Synechocystis* Δ*sigF* foram crescidas em biorreatores com arejamento. Posteriormente, o polímero foi isolado e o seu conteúdo em péptidos e sulfato foi manipulado. As variantes obtidas foram testadas *in vitro*, na linha celular Mewo, e *in vivo*, através do modelo da membrana corioalantóide de embrião de galinha (CAM). Os resultados obtidos demonstraram que a introdução do arejamento nas culturas de *Synechocystis* Δ*sigF* aumentou a taxa de crescimento celular, não afetando, contudo, a quantidade de RPS produzida por célula. No entanto, a quantidade total de polímero obtida após 30 dias de cultura em biorreatores com arejamento foi 1,5 vezes mais elevada do que na cultura crescida em matrazes com agitação orbital, indicando que este sistema de biorreatores é adequado para aumentar a produção do polímero Δ*sigF*. Anteriormente, foi também demonstrado *in vitro*, que o polímero Δ*sigF* com conteúdo reduzido em péptidos (menos 40%) tem atividade antitumoral mais forte do que o polímero Δ*sigF* não modificado na linha celular Mewo. Neste trabalho, a metade da concentração inibitória máxima (IC_{50}) da variante do polímero com conteúdo reduzido em péptidos foi determinada, e a atividade antitumoral de ambas as variantes foi validada *in vivo*, através do modelo CAM. Para além disso, espectroscopia de infravermelho com transformada de Fourier (FTIR)

permitiu identificar os grupos funcionais que podem estar relacionados com a bioatividade destes polímeros.

Em resumo, este tipo de polímeros extraídos de cianobactérias podem constituir uma plataforma promissora para o desenvolvimento de um bioproduto adequado para o tratamento de tumores, nomeadamente de melanomas.

Palavras-chave: cianobactérias; substâncias poliméricas extracelulares (EPS); polímero Δ*sigF*, variantes do polímero, atividade antitumoral (*in vitro*; modelo *in vivo* CAM).

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List of abbreviations

ABC- ATP binding cassette transporter

ANOVA- Analysis of variance

BSA- Bovine serum albumin

CAM- Chicken Embryo Chorioallantoic Membrane

Chl *a*- Chlorophyll *a*

DMEM - Dulbecco's Modified Eagle Medium

DNA- Deoxyribonucleic acid

DW- Dry weight

EDD- Embryonic development day

EPS- Extracellular Polymeric Substances

FBS- Fetal bovine serum

FTIR- Fourier transformed infrared spectroscopy

HCl- Hydrogen chloride

HEP- Heterocyst envelope polysaccharide

HPS- Hormogonium-specific polysaccharides

IC_{50}- Half maximal Inhibitory Concentration

NtcA- Nitrogen-control transcription factor

OD- Optical density

PCC- Pasteur culture collection

PCC-M- Pasteur culture collection Moscow sub-strain

PCR- Polymerase chain reaction

PHB- Polyhydroxybutyrate

r. p. m- Revolutions per minute

RPS- Release polysaccharides

RT- Room temperature

sigF, J- Alternative sigma factor F, J

TCA- Trichloroacetic acid

WHO- World health organization

UV- Ultraviolet

wt- Wild-type

Introduction

1.Cyanobacteria

Cyanobacteria represent a widespread group of autotrophic Gram-negative bacteria capable of performing oxygenic photosynthesis (Sharma et al. 2013). This ability allowed cyanobacteria to shape the geochemistry of the planet in the Precambrian, being responsible for the oxygenation of the primitive Earth's atmosphere (Schopf 2002). Afterwards, the cyanobacterial endosymbiotic relationship with a non-phototrophic host resulted in the origin of the chloroplast, leading to the emergence of primitive eukaryotic photoautotrophs and, consequently, the evolution of photosynthetic algae and plants (Ku et al. 2015). Furthermore, cyanobacteria display a crucial role in the global primary production, being important players in the biogeochemical cycles of oxygen, carbon, and nitrogen (Sharma et al. 2013). These microorganisms constitute a very morphologically diverse group that comprise unicellular, colonial, and filamentous forms. Some filamentous strains are capable of cellular differentiation, developing heterocysts (cells specialized in nitrogen fixation), akinetes (resting cells for survival under environmental stress) or motile hormogonia (short and motile chains of cells for short-distance dispersal and host infection). They can be found as free-living organisms, aggregates, microbial mats and biofilms, or live in symbiotic association with other organisms, providing nitrogen and/or carbon to their host (Rippka et al. 1979, Whitton 1992, Castenholz et al. 2001).

The long cyanobacteria evolutionary history is one of the main causes for their cosmopolitan distribution and the extensive variety of their ecological niches, ranging from saline or freshwater to terrestrial and extreme environments, being characterized as one of the most successful and oldest life forms. In fact, cyanobacteria are capable of tolerating ecological changes in their habitats and adapt to extreme environmental conditions, such as deserts, high or low temperatures, hypersaline waters, high UV radiation and desiccation (Pereira et al. 2009, Sharma et al. 2013). Recently, cyanobacteria become a rich source of valuable biomacromolecules and other metabolites, which make them attractive platforms for industrial and biotechnological applications (reviewed in Dittmann et al. 2001, Singh et al. 2017). Moreover, their easy genetic manipulation and high growth rates compared to other photosynthetic organisms increase their commercial potential, becoming promising candidates for multiples sectors, such as wastewater treatment, biofertilization and production of biofuels, as well

as cosmetic and pharmaceutical industries, due to their bioactive compounds with antioxidant and anti-inflammatory properties, for example (Singh et al. 2017, Garlapati et al. 2019). Recently, biotechnological advances have focus on optimize the production of high value products/compounds, namely biofuels and commodity chemicals, through genetic/metabolic engineering as a sustainable alternative to synthetic ones (Ducat et al. 2011, Carroll et al. 2018).

2. Extracellular polymeric substances (EPS)

2.1. Main features and roles

The extracellular polymeric substances (EPS) are biomacromolecules secreted by both unicellular and multicellular organisms, and, due to their composition and physicochemical properties, have become more attractive for several industrial and biomedical applications (Wotton 2004, Gunn et al. 2016, Moradali and Rehm 2020).

Long before the acquisition of this term, the EPS were already the target of several studies, being reported in 1875 by Thomas Henry Huxley when he studied mud from the Atlantic seafloor and discovered an albuminous slime that formed a continuous mat of living protoplasm (Rehbock 1975). At that time, Huxley was convinced that it was a primordial slime and decided to designated it as *Bathybius haeckelii*, since it was in agreement with the Ernst Haeckel's hypothesis, in "The History of Creation", that postulated the origin of life from a primordial slime (Flemming 2016). Nevertheless, years later was demonstrated that this "primordial slime" was, in fact, a calcium sulphate precipitate, which resulted from the alcohol used to preserve the samples (Rehbock 1975, Flemming 2016). Later on, the EPS were described as macromolecules mainly formed by polysaccharides that can be composed by one type of monosaccharide (homopolysaccharides) or various sugar residues (heteropolysaccharides) (Flemming and Wingender 2010, Gunn et al. 2016). The EPS can play several physiological roles that are crucial to the producing strains, such as protection against biotic and abiotic stress, acting as a direct response to natural environmental variations (Wotton 2004, Flemming 2016). Furthermore, they can increase resistance against desiccation, due to their high capacity of water retention, and can play a role in infection processes, conferring resistance to nonspecific and specific host defense mechanisms (Laspidou and Rittmann 2002, Flemming and Wingender 2010, Donot et al. 2012). Besides

protection, more functions have been attributed to EPS, such as contribution to the formation, functional integrity and stability of biofilms, cell adhesion, chelation of metal and ions, motility, regulation of molecules diffusion, nutrient entrapment, cell-cell communication (e.g. as quorum sensing molecules) and, indirectly, horizontal gene transfer between the cells (extensively reviewed in Wotton 2004, Flemming and Wingender 2010, Donot et al. 2012, Flemming 2016).

2.2. Biosynthesis

Several studies reported the possible conservation of the EPS biosynthetic mechanisms in Gram-negative and Gram-positive bacteria (Low and Howell 2018). Generally, the EPS production is a 3-step process taking place in different cell compartments: (i) in the cytoplasm occurs the activation of monosaccharides into sugar nucleotides; (ii) the repeating units are transferred onto a carrier (typically lipidic), through the action of glycosyltransferases at the plasma membrane; (iii) then the assemble and/or polymerization of the units occurs, and the produced polymer is exported to the cell surface. The assembly and export of the EPS usually follow one of three possible mechanisms: ABC transporter-, Synthase- and Wzy-dependent pathways (Fig. 1). In the case of the ABC transporter-dependent pathway, all reactions involved in the polymerization occur on the cytoplasmic face of the plasma membrane. Afterwards, the polysaccharide is carried along the plasma membrane by an ABC transporter (KpsM/KpsT) and exported through the outer membrane by a transmembrane complex formed by KpsE and KpsD proteins. In the Synthase-dependent pathway, the machinery involved may differ significantly on the polymer produced (Low and Howell 2018). Generally, a synthase (e.g. Alg8 or BcsA) is responsible for the simultaneous polymerization and export of the polymer across the plasma membrane. In the periplasm, other proteins are able to modify the polymer or degrade polymer surplus and, in the final step, the polysaccharide is guided and protected from degradation by two independent proteins (e.g. AlgK and AlgE) or only one protein with both functions (e.g. BcsC) (Pereira et al. 2015, Whitfield et al. 2015). In the Wzy-dependent pathway, after activation of monosaccharides, lipid-linked repeating units are translocated to the periplasmic face of the plasma membrane through Wzx and the polymerization is catalyzed by Wzy. Finally, the translocation of the polymer through the cell envelope is controlled by the

transmembrane complex Wza/Wzc, being the phosphorylation state of Wzc controlled by the phosphatase Wzb (Islam and Lam 2014, Pereira et al. 2015).

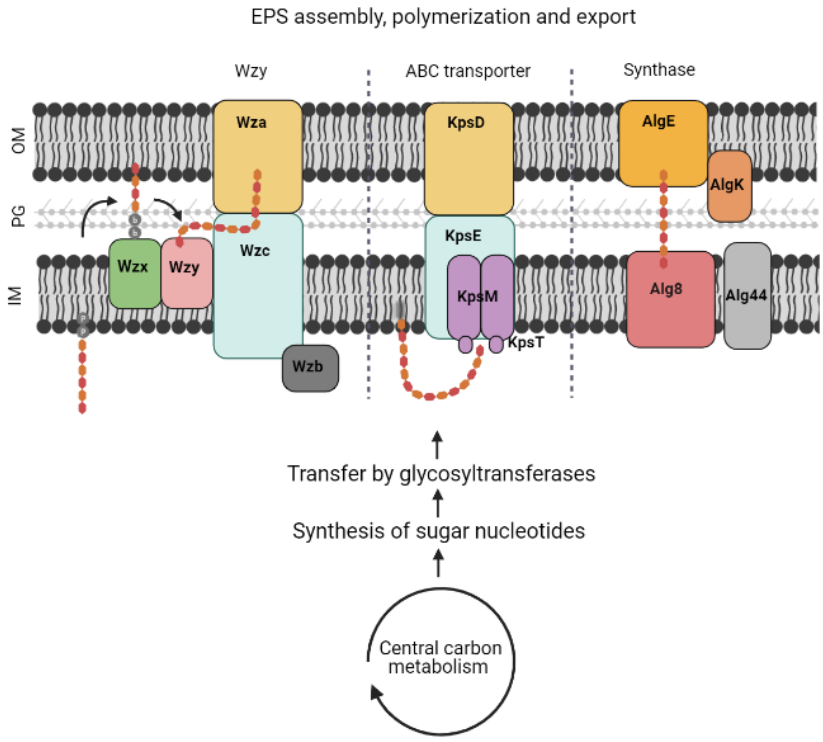

Figure 1. Schematic representation of the main events leading to the production of bacterial extracellular polymeric substances (EPS). The EPS assembly, polymerization and export may occur by one of the three main mechanisms: the Wzy-, ABC transporter- or Synthase-dependent pathways. IM- inner membrane; PG- peptidoglycan; OM- outer membrane. Adapted from Pereira et al. (2019).

2.3. Cyanobacterial EPS

Many cyanobacterial strains are able to produce EPS, mainly composed by heteropolysaccharides. These polymers can be covalently linked or loosely attached to the cell surface that according to their consistency and thickness can be referred as capsules (thick and slimy layer closely associate with the cells), sheaths (thin and dense layer loosely covering the cells) or slimes (mucilaginous material with looser association with the cells); or be released into the surrounding environment (released polysaccharides - RPS) (Rossi and De Philippis 2016).

In agreement to other bacteria, the cyanobacterial EPS can play a defensive and protective role against adverse environmental conditions (Wotton 2004). In fact, it was

described the presence of UV absorbing substances in the sheaths of several cyanobacterial strains, which confirms the role of the EPS in protecting cyanobacterial cells from the harmful effects of UV radiation (Garcia-Pichel and Castenholz 1991). In addition, the cyanobacterial EPS can be involved in the gliding motility of hormogonia (Khayatan et al. 2015), prevention of cell damage from desiccation (Danin et al. 1998), participation in the sequestration of metals and nutrients (Parker et al. 1996, Mager and Thomas 2011) and in biofilm formation (Rossi and De Philippis 2015), and in the protection of nitrogenase against the negative effects of oxygen, through the formation of an heterocyst envelope polysaccharide (HEP) layer (Muro-Pastor and Hess 2012).

The identification of the physiological conditions that influence the synthesis of the EPS is crucial for the optimization of their productivity. EPS production is described as strain- and growth-dependent. Nevertheless, over the years, several studies have been indicating a vast number of environmental factors that can affect the amount of cyanobacterial EPS produced, as well as influence their composition, structure and physicochemical properties (Pereira et al. 2009). Light (intensity and quality) seems to be one of the major factors influencing the cyanobacterial EPS production. The exposure to continuous light or high-light intensities lead to higher production of EPS by some strains (Lupi et al. 1994, Mota et al. 2013), whilst in other cases, specific light wavelengths were responsible for their significant enhancement (Ehling-Schulz et al. 1997, Han et al. 2014). The nutrient availability (e.g. of nitrogen, phosphate, and sulphate) and its cell ratio (e.g. C:N ratio) can also affect the biosynthesis of the EPS. It is well established that higher availability of nitrogen and/or carbon increase the EPS production, however N starvation can also be responsible for higher yield of EPS production (Otero and Vincenzini 2003, Kumar et al. 2007). Accordingly, studies revealed that starvation or limiting availability of other nutrients, such as manganese, phosphorous, potassium or sulfur, resulted in a maximization in EPS production (Markou et al. 2012). Moreover, aeration can stimulate EPS production by improving light penetration and nutrients availability, or by promoting a physical separation of the EPS from the cells surface (Moreno et al. 1998, Su et al. 2007).

Despite this knowledge, the data available to understand the intricate machinery and the specific pathways of cyanobacterial EPS biosynthesis is still very limited. Recent studies suggest a much more complex scenario regarding the molecular machinery involved in the assembly and export of the cyanobacterial EPS compared to other bacterial EPS. A phylum-wide analysis showed that many cyanobacteria contain genes encoding proteins putatively involved in each of the three aforementioned pathways, but

not the complete set defining one pathway (Pereira et al. 2015). This suggested that the cyanobacterial EPS production does not follow a single pathway and/or more elements are involved, being the genes under different regulatory mechanisms. Herewith, concerning the high number of genes involved in cyanobacterial EPS production, and that some may encode proteins with redundant function, it is expected that an intricate regulatory network might operate to control the EPS biosynthesis. Up to date, the majority of the studied regulatory elements associated to cyanobacterial EPS were reported in filamentous strains, being most of them related with the production of the heterocyst HEP-layer. For example, in *Anabaena* PCC 7120, the nitrogen-control transcription factor (NtcA) is indirectly involved with the control of EPS biosynthesis (López-Igual et al. 2012). Furthermore, it is expected that the alternative sigma factors may play an important role in EPS production control, since they are known to be involved in regulation of acclimatation responses and survival in diverse environmental conditions (Feklístov et al. 2014). It was demonstrated, also in *Anabaena* PCC 7120, that the alternative Group 3 sigma factor SigJ, which is associated with desiccation tolerance, plays a role in EPS production (Yoshimura et al. 2007, Srivastava et al. 2016). In the case of *Nostoc punctiforme*, SigJ (as well as SigF) is involved in hormogonium development control, activating genes associated with synthesis and secretion of hormogonium-specific polysaccharide (HPS) (Gonzalez et al. 2019). Recently, the impact of SigF in motility control, production of RPS/EPS, vesiculation and protein secretion in *Synechocystis* sp. PPC 6803 was reported (Flores et al. 2019a).

2.3.1. Distinct features of cyanobacterial EPS

Cyanobacterial EPS exhibit unique features when compared to EPS produced by other microbial sources, such as:

(i) **high number of different monosaccharides** (up to 13), which result in a variety of linkage types and lead to complex architectures and structures (Pereira et al. 2009). Whilst the EPS produced by other bacteria generally contain less than four monosaccharides, most of the cyanobacterial EPS described contain six or more different types of monomers (Rossi and De Philippis 2016). Up to date, have been found in cyanobacterial EPS, hexoses (fructose, galactose, glucose and mannose), pentoses (arabinose, ribose and xylose) and deoxyhexoses (fucose and rhamnose). Furthermore, they frequently have **one or two hexoses/uronic acids** (glucuronic and galacturonic

acid) in their composition (Pereira et al. 2009). Glucose is the monosaccharide most frequently found in cyanobacterial EPS, however, in some species, the presence of arabinose, fucose, galactose or mannose occur in higher concentrations than glucose (Parikh and Madamwar 2006, Rossi and De Philippis 2016).

(ii) **sulphate groups**, which is extremely rare among EPS from bacteria, but common between EPS produced by archaea and eukaryotes. Together with the presence of uronic acids and the ketal-linked pyruvate groups, the sulphate groups are important to confer strong anionic charge and a "sticky" behavior to the EPS (Sutherland 1994, Arias et al. 2003).

(iii) **peptides**, usually enriched in alanine, glycine, valine, leucine, isoleucine, and phenylalanine (Kawaguchi and Decho 2002). The presence of proteins enriched with aspartic and glutamic acids was also observed in some cases (Flaibani et al. 1989, Garozzo et al. 1998). These polypeptides, together with deoxysugars and ester-linked acetyl groups, confer high levels of hydrophobicity and, consequently, the emulsifying and rheological properties of the EPS (Neu et al. 1992, Pereira et al. 2009).

(iv) **unusual sugars**, such as methyl sugars (e.g. 4-O-methyl rhamnose, 3-O-methyl glucose, 2,3-O-methyl rhamnose and 3-O-methyl rhamnose) and/or amino-sugars (glucosamine and galactosamine and their N-acetyl derivates) (Hu et al. 2003).

(v) **high-molecular mass fractions**, reaching values of more than 2 MDa in some cases. This feature contributes to modify the rheological properties of water by acting as thickening agents, for example (Xu and Zhang 2016).

Furthermore, other non-carbohydrates constituents (e.g. pyruvate and acetate) have been identified in cyanobacterial EPS, for example in members of *Cyanothece* (De Philippis and Vincenzini 1998) and *Nostoc* (De Philippis et al. 2000) genus. Compared with other microbial polysaccharides, the cyanobacterial EPS are still less characterized. Despite the intensive research, the structure of cyanobacterial EPS is hindered by their complex nature, however, their particular features and structural complexity also make these polymers attractive platforms for biotechnological/biomedical applications (Garozzo et al. 1998, Gloaguen et al. 1999, Helm et al. 2000, Shah et al. 2000, Volk et al. 2006).

2.3.2. Biotechnological application of cyanobacterial EPS

Cyanobacterial EPS are biodegradable and biocompatible macromolecules, which make them promising sustainable and eco-friendly substitutes to petrochemical-derived polymers available in the market (Rehm 2010, Donot et al. 2012). Moreover, the industrial production of cyanobacterial EPS confers important advantages compared to biopolymers derived from plants or algae since: (i) the cyanobacterial producing strains have only minimal nutritional requirements, preventing the need of expensive EPS precursors/substrates and allowing a better control of growth/production and low variability of the final product; (ii) usually exhibit higher growth rates, which accelerate the production process; (iii) can be easily manipulated by genetic engineering, allowing to obtain polymers with specific properties for high-value applications; (iv) the culture conditions can be easily modified in order to optimize the EPS production; (v) the EPS are commonly secreted by the cells, which facilitates the isolation process; (vi) and have particular physicochemical properties, which can lead to interesting and novel functionalities (Selbmann et al. 2002, Freitas et al. 2011, Rütering et al. 2016). Overall, cyanobacterial EPS represent a low-cost raw material adaptable to a high number of distinct fields.

The main areas of biotechnological application of cyanobacterial EPS have been bioremediation, cosmetics, pharmaceutics, food, painting industry and soil stabilization (reviewed in Singh et al. 2017). Recently, research has focused on cyanobacterial EPS potential for biomedical applications, such as the development of innovative biomaterials (e.g. scaffolds, drug carriers and coatings) (Leite et al. 2017, Costa et al. 2019, Costa et al. 2021, Matinha-Cardoso et al. 2021). Additionally, a vast array of biological activities have been described for cyanobacterial EPS, such as antitumor (Yue et al. 2012, Ou et al. 2014), immunostimulatory (Løbner et al. 2008), anticoagulant (Yamamoto et al. 2003), antioxidant (Parwani et al. 2014), antibacterial (Najdenski et al. 2013), antifungal (Najdenski et al. 2013) and antiviral (Hayashi et al. 1996, Ahmadi et al. 2015, Mader et al. 2016, Reichert et al. 2017). These distinct bioactivities are attractive for several biomedical applications and may be associated with their compositional and structural features, such as the accessibility/presence of amino-sugars, sulfate groups and uronic acids, low molecular mass fractions, and hydrophobic nature. Nevertheless, the majority of the studies only infer the association between the features of cyanobacterial EPS and their bioactivities, based on physiochemical comparisons to EPS from other sources (Gacheva et al. 2013, Flamm and Blaschek 2014). For example, by comparison with

other sulfated EPS, it has been described that the high sulfate content of cyanobacterial EPS may be strongly involved in antiviral (Aleksandar et al. 2010), antioxidant (Parwani et al. 2014), anticoagulant (Majdoub et al. 2009) and antitumor (Ruiz-Ruiz et al. 2011) activities.

2.4. Cyanobacterial EPS as antitumor agents

Cancer is the second major cause of death worldwide, with an estimation of 19.3 million new cases and 9.98 million of deaths in 2020. Based on the GLOBOCAN project in 2020, it is expected more than 30 million new cancer cases and 16 million deaths by 2040 (Ferlay et al. 2020). These predicted high numbers increase the demand of early cancer detection, such as screening programs, and the development of advanced and innovative therapies and treatments. Due to the limitations and negative side effects of chemotherapy and radiotherapy, more researchers are focused in alternative therapeutics that are clinically effective and less expensive, namely anticancer agents from natural sources (Cragg and Newman 2018, Khan et al. 2019). Several studies have been showing that biopolymers of polysaccharidic nature, including the cyanobacterial EPS, may be valuable antitumoral agents, by preventing tumor development and inhibiting proliferation, invasion, adhesion, metastasis and/or angiogenesis (Zong et al. 2012). Furthermore, studies with EPS from different sources have been shown that five types of antitumor molecular mechanisms may be triggered: (i) cell cycle arrestment; (ii) activation of the mitochondrial-mediated apoptotic pathway; (iii) production and activation of nitric oxide (NO) pathway; (iv) immunomodulatory pathways; (v) other specific mechanisms with pathways not define yet, such as production of reactive oxygen species and inhibition of galectin 3 and topoisomerase (Zong et al. 2012, Khan et al. 2019).

Despite the very limited number of studies, the antitumor activity potential of cyanobacterial cell crude extracts containing EPS or their isolated EPS have already been described (Yue et al. 2012, Li et al. 2018). However, most of these studies are based on the antioxidant and antiproliferative (Li et al. 2011, Yue et al. 2012, Gacheva et al. 2013, Ou et al. 2014, Li et al. 2018), antimigration (Gloaguen et al. 1999), and anti-invasion (Mishima et al. 1998) activities of cyanobacterial EPS, and not directly correlated to their antitumor activity. Moreover, the induction of apoptosis trough caspase-3 activation in tumor cells was reported in studies with EPS from *Aphanothece*

halophytica (Ou et al. 2014), *Nostoc sphaeroides* (Li et al. 2018), and *Synechocystis* sp. PPC 6803 (Flores et al. 2019b). Nevertheless, *in vivo* studies performed with cyanobacterial EPS are still very scarce, and only two studies were reported using tumor cell-transplanted mice (Zheng et al. 1994, Mishima et al. 1998). In this context, intensive research efforts are still required to unveil and understand the antitumor molecular mechanisms and the EPS compositional/structural features that are related to their antitumor activity.

2.4.1. *Synechocystis* EPS as antitumor agent

Synechocystis sp. PCC 6083 is a well characterized and genetic amenable unicellular cyanobacterium that was considered the "green *E. coli*" (Branco dos Santos et al. 2014). Therefore, despite being a moderate EPS-producer, this strain has been used to study the genes involved in EPS production (Pereira et al. 2019, Santos et al. 2021). Recent studies reported that *Synechocystis* sp. PCC 6083 Group 3 sigma factor SigF knockout mutant (Δ*sigF*) releases up to 4-fold more polysaccharides than the wild-type (Flores et al. 2019a). The Δ*sigF* polymer was shown to have a strong antitumor activity, by reducing the cell viability in a time- and dose-dependent manner in three human tumor cell lines: melanoma (Mewo), thyroid carcinoma (8505C) and ovarian carcinoma (A2780) (Flores et al. 2019b). In addition, high levels of apoptosis of the Δ*sigF* polymer were reported, reaching up to ~40%, which was correlated to the increased levels of caspase-3 and p53, showing the induction of mitochondrial-mediated apoptotic pathway. Furthermore, these high levels of apoptosis suggest that Δ*sigF* polymer induced one of the highest levels of apoptosis observed among natural polymers (Ruiz-Ruiz et al. 2011, Liu et al. 2016, Li et al. 2018, Flores et al. 2019b). The polymer isolated from Δ*sigF* culture was also extensively characterized, being detected high sulfate (≈12% w/w) and protein (≈27% w/w) contents, and four fractions with distinct molecular mass, being the >800 kDa the most abundant (Flores et al. 2019a). However, the role of this constituents in the polymer antitumor activity is still unclear.

Aims

Previously, it was demonstrated by the research group, in which this work took place, that an extracellular carbohydrate polymer released by a mutant of a unicellular cyanobacterium (*Synechocystis* Δ*sigF*) exhibits a strong antitumor activity against several human tumor cell lines. Therefore, the major aim of this study was to gain an insight into the polymer features that are relevant for its antitumor activity. For this purpose, the polymer peptide and/or sulfate contents were manipulated originating different variants that were tested on the human melanoma cell line (Mewo). In addition, the antitumor activity of the most promising variant was evaluated *in vivo*, using the chicken embryo chorioallantoic membrane (CAM) model.

Materials and Methods

1. Cyanobacterial strains and culture conditions

The cultures of *Synechocystis* sp. PCC 6803 wild-type (sub-strain PCC-M) and its Δ*sigF* knockout mutant (Huckauf et al. 2000, Trautmann et al. 2012)(Huckauf et al. 2000, Trautmann et al. 2012) were grown in Erlenmeyer flasks containing BG11 medium (550 mL of culture) (Rippka et al. 1979) at 30 ºC under 12 h light (50 μE m^{-2} s^{-1})/12 h dark regimen, with orbital shaking at 150 r.p.m. The Δ*sigF* cultures were also grown in Erlenmeyer flasks containing BG11 medium enriched with sulfur (supplemented with 1 g L^{-1} $CaSO_4 \cdot 2H_2O$ and with less 0.85 g L^{-1} $CaCl_2 \cdot 2H_2O$ to avoid surplus of calcium) in the same culture conditions. To study the optimization of the *ΔsigF* polymer production, *Synechocystis ΔsigF* was grown in bioreactors (550 mL of culture) at 30 °C, under a 12 h light (50 μE m^{-2} s^{-1})/12 h dark regimen, with continuous aeration (1.2 L min^{-1}). The *ΔsigF* mutant was maintained in BG11 medium supplemented with kanamycin (100 μg mL^{-1}), while the experiments were performed in the absence of selective pressure.

2. DNA extraction and confirmation of the segregation of the Δ*sigF* mutant

Cyanobacterial genomic DNA was extracted as described by Ferreira et al. (2018) and DNA concentration was determined using a Nanodrop ND-1000 (Nanodrop Technologies Inc., USA). Complete segregation of the mutants was confirmed by PCR amplification using the oligonucleotide primers listed on Table 1, as reported by Flores et al. (2019a). PCR reactions were performed using a thermal cycler (MyCycler™, Bio-Rad laboratories Inc., Hercules, CA, USA) following procedures previously described (Tamagnini et al. 1997). The PCR profile included an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 54 °C for 1 min, 72 °C for 75 s, and a final extension at 72 °C for 7 min. The PCR products were separated by agarose gel electrophoresis (Sambrook and Russell 2001).

Table 1. List of oligonucleotides used in this work to confirm *Synechocystis* Δ*sigF* segregation.

Primer name	Sequence (5´- 3')	Reference
Slr1564.5O	GGGTGGTTATCAACAGCAAGCCCAGCAAAT	(Flores et
Slr1564.3O	GAGATTGTGGAGGTAACCTGCACTCTGGCT	al. 2019a)

3. Light microscopy

Cells were observed using an Olympus X31 light microscope (Olympus, Spain) and micrographs were acquired with an Olympus DP25 camera and the Cell B software (Olympus, Spain). Cells were also stained with Alcian Blue (0.5% w/v in 50% ethanol) for the visualization of acid carboxylated and sulfated polysaccharides (Thornton et al. 2007).

4. Growth assessment

Growth measurements were performed by monitoring optical density, dry weight, and the chlorophyll *a* content. Optical density (OD) was measured spectrophotometrically at 730 nm according to Anderson and McIntosh (1991). For the determination of the dry weight (DW), 5 mL of culture were dried at 60 ºC until a constant weight was reached. The content of chlorophyll *a* (chl *a*) was extracted using 90% (v/v) methanol and determined spectrophotometrically as reported by Meeks and Castenholz (1971). All the measurements were performed with three technical replicates.

5. Determination of total carbohydrate content and released polysaccharides (RPS)

The content of total carbohydrates and RPS in cyanobacterial cultures was determined by the phenol-sulfuric acid method (Dubois et al. 1956), as previously reported by Mota et al. (2013). All experiments were performed with three technical replicates.

6. Polymer isolation

The Δ*sigF* polymer was isolated according to Flores and Tamagnini (2019). Briefly, the *Synechocystis* Δ*sigF* cultures were dialyzed (12–14 kDa of molecular weight cut-off; Medicell International) against a minimum of 10 volumes of deionized water for 48 h with continuous stirring. Then, the cultures were centrifuged at 20,000 x *g* for 25 min at 8 °C, and 2 volumes of 96% ethanol were added to the supernatant. After an incubation at 4 ºC overnight, the suspension was centrifuged at 20,000 x *g* for 25 min at 6 °C, and the supernatant was discarded. The pellet was resuspended in 1 mL of autoclaved type II water and lyophilized. The dried polymer was stored at room temperature (RT) until further use.

7. Quantification of protein and sulphate contents

The lyophilized polymers were resuspended in deionized water and the peptide content was quantified using the Lowry method (Lowry et al. 1951). The sulfate content was determined by hydrolyzing 2 mg of the Δ*sigF* polymer with 1 mL of 2 M HCl for 5 h at 110 °C, and the quantification was performed with Sulfate Assay Kit (MAK 132, Sigma-Aldrich Co., MO, USA), according to the manufacturer's guidelines.

8. Peptide removal and analysis

The peptide content of the polymer was removed by trichloroacetic acid (TCA) precipitation. Briefly, an aqueous polymer solution (5 mg mL^{-1}) was incubated with 15% TCA, for 15 min at RT, and then centrifugated at 11000 x *g* for 20 min at 4 ºC. Then, the supernatant was dialyzed against a minimum of 10 volumes of deionized water for 48 h with continuous stirring and lyophilized, as performed for the polymer isolation. The treatment efficiency was assessed by measuring the peptide content before and after the TCA precipitation using the Lowry method, as described above.

9. Human tumor cell lines and culture conditions

The human melanoma cell line Mewo (kindly given by Prof. Marc Mareel, Department of Radiotherapy and Nuclear Medicine, Ghent University Hospital, Belgium) (Pópulo et al. 2015) was cultured in DMEM culture medium with stable glutamine (Capricorn Scientific, Germany) and supplemented with 10% fetal bovine serum (FBS, GIBCO, Invitrogen, UK), 1× penicillin/streptomycin (Biowest, France) and 1.25 µg mL^{-1} amphotericin B (Corning, USA). Cells were maintained in a humidified incubator at 37 °C with 5% CO_2. The cell line was authenticated following genotyping at Institute for Research and Innovation in Health (i3S) Genomics Core Facility (Porto, Portugal) using the PowerPlex® 16 HS System (Promega, USA) and according to DNA profiles available at ATCC and ECACC STR profiles database. Cells were also confirmed to be free of mycoplasma contamination following at Genomics Core Facility (Porto, Portugal)

10. Cell viability assays

Cell viability analysis was carried out performing a resazurin based assay, using PrestoBlue™ Cell Viability Reagent as described in Pópulo et al. (2015). Briefly, cells were plated on 96-well plates (1×10^4 cells/well) and allowed to adhere for 24 h at 37 °C. Cells were then treated with: supplemented medium (Blank); *Synechocystis* Δ*sigF* polymer and respective variants resuspended in non-supplemented medium at concentrations ranging from 0.0875 to 1.5 mg mL^{-1} or with polymer vehicle (supplemented medium containing the equivalent amount of non-supplemented medium used in the polymer solutions). Following 48 h treatment, cells were washed three times with non-supplemented medium and further incubated for 45 min with PrestoBlue™ reagent (Life Technologies, EUA) previously diluted 1:10 in supplemented medium. Fluorescence was then measured (excitation 560 nm; emission 590 nm) using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments Inc., EUA). Five technical and four biological replicates were analyzed. Cell viability was determined by analyzing the mean fluorescence values obtained for each sample as percentage of the values obtained for control cells (Blank cells), after removing the background values (medium only).

11. Fourier transformed infrared spectroscopy (FTIR)

For Fourier transformed infrared spectroscopy (FTIR), 2 mg of polymer were mixed with 200 mg of KBr and the powders pressed at 8 tons for 1 min, using an automatic press. FTIR spectra of the polymer were acquired using a Frontier FTIR spectrometer (PerkinElmer, USA) from 4000 to 400 cm^{-1} and with 4 cm^{-1} resolution.

12. *In vivo* chicken embryo chorioallantoic membrane (CAM) angiogenesis and tumor growth assay

The effect of the Δ*sigF* polymer variants on the angiogenic response and tumor growth of Mewo cells was evaluated *in vivo,* using the chicken embryo chorioallantoic membrane (CAM) model as previously described (Ferreira et al. 2016, Leite et al. 2020). According to the European Directive 2010/63/EU, ethical approval is not required for experiments using embryonic chicken. Correspondingly, the Portuguese law on animal welfare does not restrict the use of chicken eggs. Briefly, fertilized chick (*Gallus gallus*) eggs were incubated horizontally at 37.5 °C in a humidified atmosphere and referred to embryonic development day (EDD 0). On EDD3, 2 mL albumen was withdrawn, and a square window was opened in the eggshell to allow the growth of the CAM detached from the shell. The window was sealed with transparent adhesive tape and the eggs were re-incubated until EDD9. At EDD9, 1×10^6 Mewo cells were resuspended in a mixture of 10 µL of 0.7 mg mL^{-1} Δ*sigF* polymer variants (unmodified Δ*sigF* or Δ*sigF* polymer with reduced peptide content) and 5 µl Matrigel™ (Corning® Inc., Bedford, MA, USA) and placed on top of the growing CAM, into a 5 mm silicon rings, under sterile conditions. As controls, the same amount of cells was used, resuspended in polymer vehicle (non-supplemented DMEM) and 5 µl Matrigel. A total of 76 eggs (29 for unmodified Δ*sigF* polymer, 28 for Δ*sigF* polymer with reduced peptide content and 19 for control), distributed through 2 independent experiments were inoculated. The eggs were resealed and further incubated for 4 days. At EDD13, embryos were euthanized by adding 2 mL of 10% neutral-buffered formalin in the top of the CAM. The rings were removed and the fixed CAMs were excised and photographed *ex ovo* under a stereoscope at 20× magnification (Olympus SZX16 coupled with a DP71 camera; Olympus Corp., Tokyo, Japan). For angiogenesis analysis, the number of new vessels

(<20 µm diameter) growing radially towards the ring area was counted in a blind manner. For tumor growth analysis, the area of CAM tumors was determined using the Cell A software (Olympus, Spain).

13. Statistical analysis

Data were plotted and statistically analyzed using GraphPad Prism version 5.0 (GraphPad Software) using analysis of variance (ANOVA), followed by Bonferroni's multiple comparisons test (growth assessment, carbohydrates production and determination of polymer's peptide content), or Dunn's multiple comparisons test (CAM assay).

Results and Discussion

1. Optimization of *Synechocystis* Δ*sigF* polymer production

Envisaging the optimization of the polymer production, cultures of *Synechocystis* Δ*sigF* were grown in bioreactors [30 °C, 12 h light (50 μE m^{-2} s^{-1})/12 h dark regimen with continuous aeration (1.2 L min^{-1})]. Since *Synechocystis* Δ*sigF* cultures were grown without selective pressure, the segregation of this mutant was confirmed by PCR amplification before each experiment (Fig. 2).

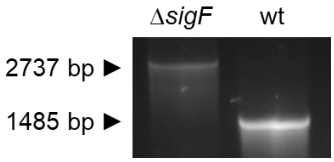

Figure 2. Confirmation of *Synechocystis* Δ*sigF* mutant segregation by PCR analysis. The PCR amplifications were performed using a specific primer pair targeting the flanking regions of the sigF gene (Slr1564.50/Slr1564.30) (for more details, see *Materials and Methods* section). Genomic DNA was extracted from *Synechocystis* PCC 6803 wild-type (wt) and *Synechocystis* Δ*sigF* cultures as described by Ferreira et al. (2018). Expected size of the PCR products: wt-1485 bp; Δ*sigF*- 2737 bp.

The growth measurements were performed by monitoring the optical density (OD), chlorophyll *a* (chl *a*) content, and dry weight (DW), and compared to Δ*sigF* cultures grown in Erlenmeyer flasks under previously established conditions [30 °C, 12 h light (50 μE m^{-2} s^{-1})/12 h dark regimen with orbital agitation]. The *Synechocystis* Δ*sigF* cells grown in bioreactors with aeration showed an increase in the growth rate (OD ~2-fold), compared to the Δ*sigF* cells grown in Erlenmeyer flasks (Fig. 3). These results are in agreement with previous studies on other cyanobacterial strains that reported faster growth rates for cultures with aeration, since this condition improve nutrient and light availability for the cells (Ogbonda et al. 2007, Monteiro et al. 2010). In contrast, the amount of total carbohydrates produced by Δ*sigF* cells grown with continuous aeration decreased compared to the cells grown in Erlenmeyer flasks (Fig.4A), and the amount of RPS did not significantly change between the two conditions (Fig. 4B). Furthermore, when the values were normalized per chl *a*, the amounts of total carbohydrates and RPS were significantly lower in Δ*sigF* cells that grow in bioreactors (Fig. 4C and 4D), indicating

that cells under continuous aeration are using their energy pools to boost their growth, rather than to carbohydrates production. This can be clearly perceive comparing the amount of polymer produced by g of DW (in Erlenmeyer flasks 84 mg of polymer per g DW; in Bioreactors 79 mg of polymer per g DW). However, the total polymer yield after 30 days of culture was 1.5-fold higher in the bioreactor compared to the Erlenmeyer flasks, so in the future this cultivation set can be utilized to increase the RPS production. Previous studies reported that aeration can increase the EPS production in some cyanobacteria strains (Moreno et al. 1998, Su et al. 2007), but it was also described that environmental conditions can affect the cells carbon/energy fluxes, redirecting it from the production of cyanobacterial EPS and other carbohydrate-rich molecules e.g. polyhydroxybutyrate (PHB) to cell growth and vice-versa (Trabelsi et al. 2009, Carpine et al. 2020).

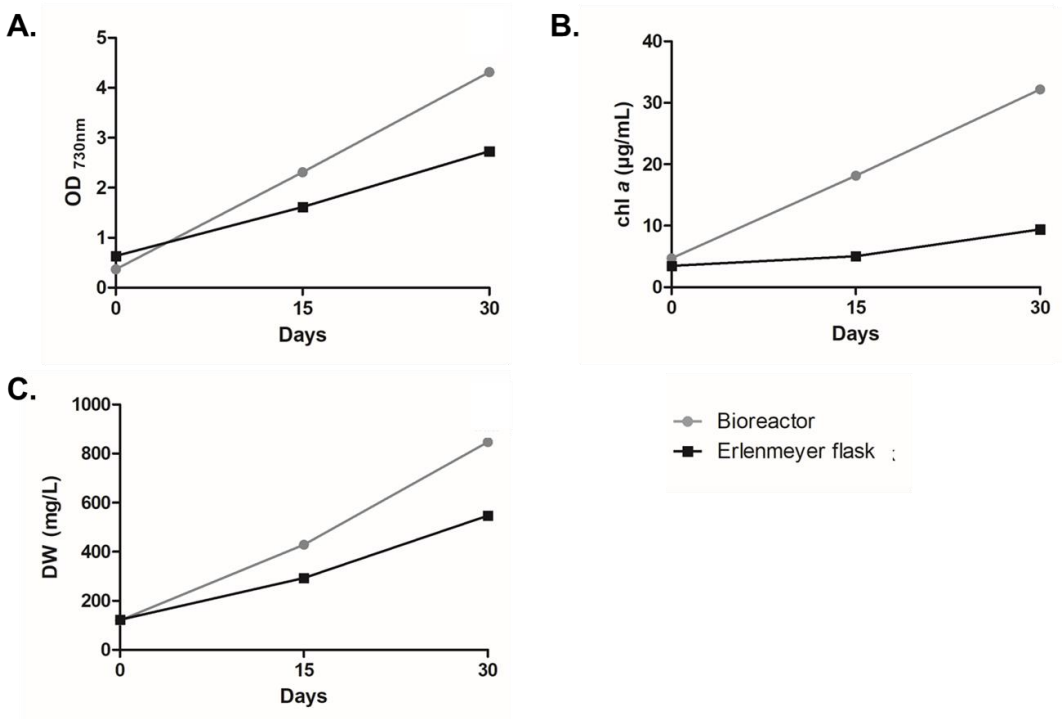

Figure 3. Growth curves of *Synechocystis* PCC 6803 Δ*sigF* cultures grown in Erlenmeyer flasks with orbital agitation (Erlenmeyer flask) and in bioreactors with aeration (Bioreactor), both at 30 °C and 12 h light (50 µE m^{-2} s^{-1})/12 h dark regimen. Growth was monitored by measuring the optical density (OD) at 730 nm (A), the chlorophyll *a* (chl *a*) content (B) and dry weight (DW) (C) (for more details, see *Materials and Methods* section).

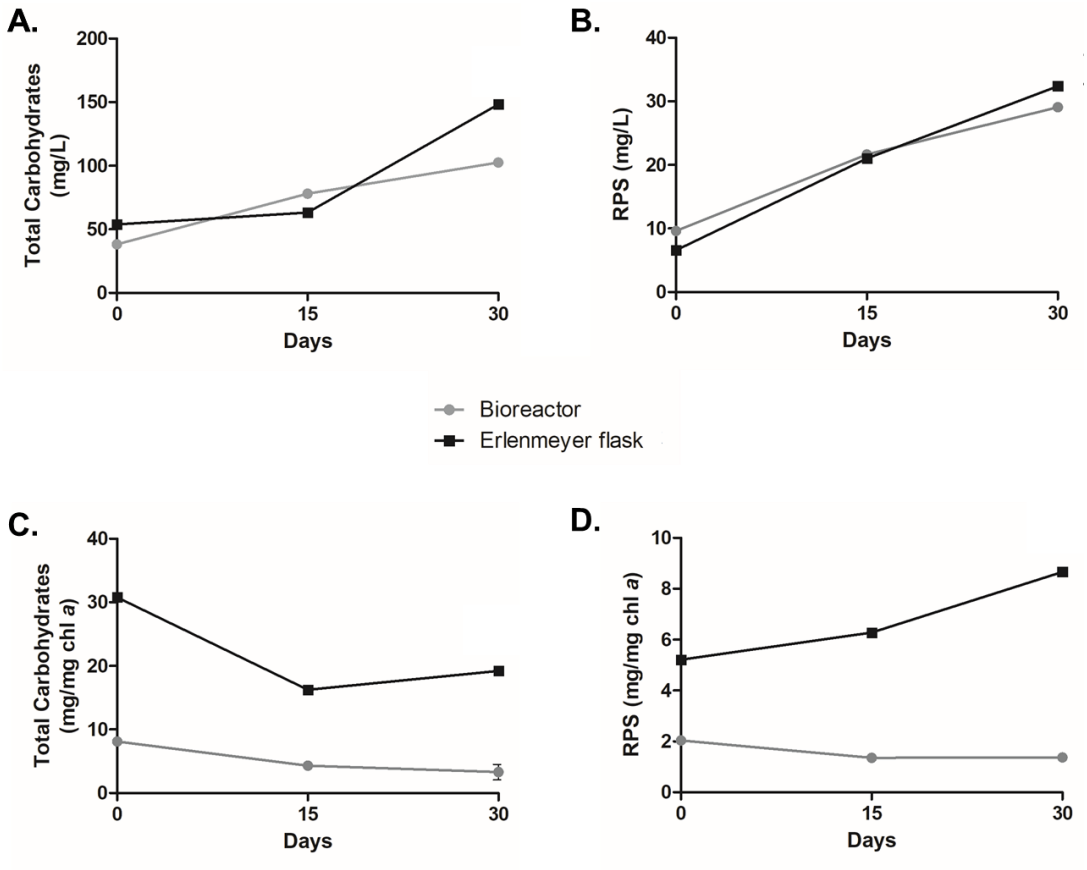

Figure 4. Total carbohydrates and released polysaccharides (RPS) of *Synechocystis* PCC 6803 Δ*sigF* cultures grown in Erlenmeyer flasks with orbital agitation (Erlenmeyer flask) and in bioreactors with aeration (Bioreactor), both at 30 °C and 12 h light (50 μE m^{-2} s^{-1})/12 h dark regimen. Total of carbohydrates and RPS production were quantified by the phenol-sulfuric method, and the values expressed as mg of carbohydrates per liter (L) of culture (A and B, respectively) or mg of carbohydrates per mg of chl *a* (C and D, respectively) (for more details, see *Materials and Methods* section).

2. Manipulation of the polymer sulfate content

It has been previously demonstrated that the Δ*sigF* polymer has high amount of sulfate (12% w/w) and peptides (28% w/w), however, is still unclear the role of these components in its strong antitumor activity (Flores et al. 2019a). Considering that a correlation between the presence of sulfate groups and cyanobacterial EPS antitumor activity was already describe in the literature (Mishima et al. 1998), the sulfate content of our polymer was manipulated. For that, *Synechocystis* Δ*sigF* cultures were grown in BG11 medium enriched with sulfate (for more details, see *Materials and Methods*). Firstly, the growth of Δ*sigF* cells grown with sulfate surplus (Δ*sigF* + SO_4) was monitored and compared to Δ*sigF* cells grown only in BG11 medium (Δ*sigF*), revealing a similar growth pattern between them (Fig. 5).

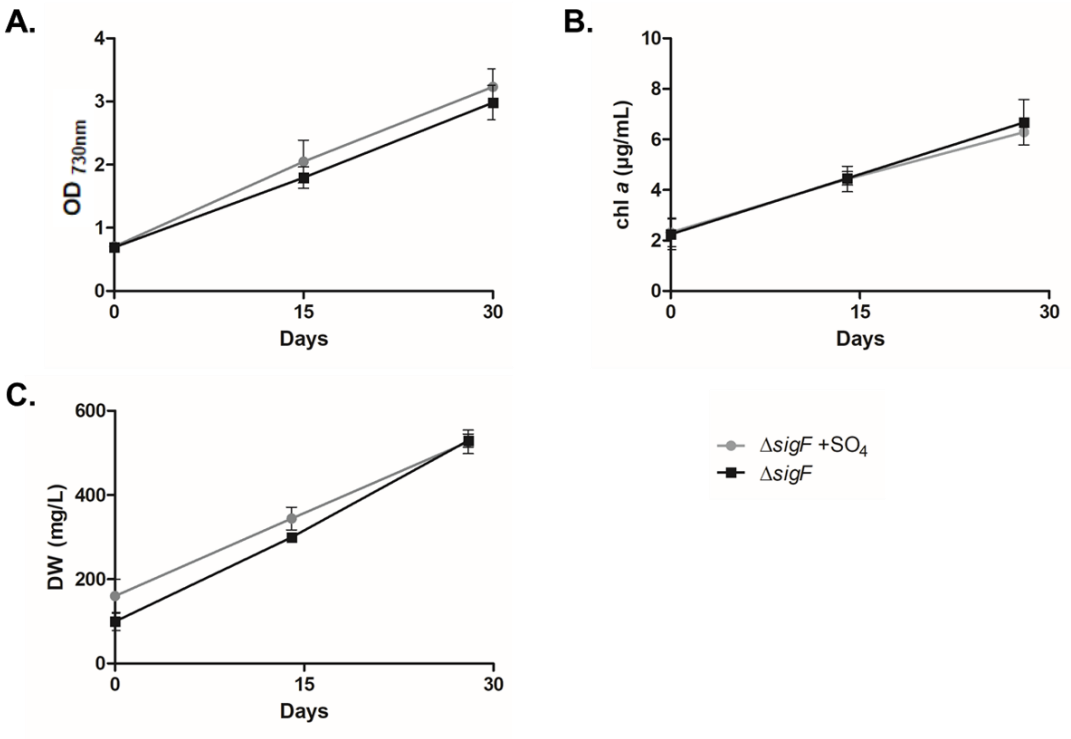

Figure 5. Growth curves of *Synechocystis* PCC 6803 Δ*sigF* cultures grown in BG11 medium (Δ*sigF*) and in BG11 medium supplemented with sulfate - 1 g L^{-1} $CaSO_4 \cdot 2H_2O$ (Δ*sigF* +SO_4). Growth was monitored by measuring the optical density (OD) at 730 nm (A), chlorophyll *a* (chl *a*) content (B) and dry weight (DW) (C) (for more details, see *Materials and Methods* section). Cultures were grown in Erlenmeyer flasks with a 12 h light (50 µE m^{-2} s^{-1})/12 h dark regimen, 30 ºC, and 150 r.p.m. orbital agitation. Experiments were made in triplicate and are represented as mean ± STD.

Additionally, the amount of total carbohydrates and amount of RPS produced by cultures with surplus of sulfate were evaluated and compared to cultures with unmodified

BG11. Regarding the total carbohydrates, the amount produced by Δ*sigF* cells grown with sulfate surplus (Δ*sigF* + SO_4) increased significantly (~2 fold-more) compared to the cells grown in unmodified BG11 (Δ*sigF*) (Fig. 6A). However, the same pattern was not observed for the RPS. The amount of RPS for the cultures grown with sulfate surplus did not change significantly, compared to the cultures grown in BG11 (Fig. 6B). This is also evident, when the values were normalized per chl *a* (Fig. 6C and 6D). These results were supported by the amount of polymer produced by g of DW, that did not show significant differences between the polymer produced by cultures grown in BG11 medium (~83 mg of polymer per g of Δ*sigF* culture DW) compared to cultures grown in medium supplemented with sulfate (~75 mg of polymer per g of Δ*sigF* culture DW).

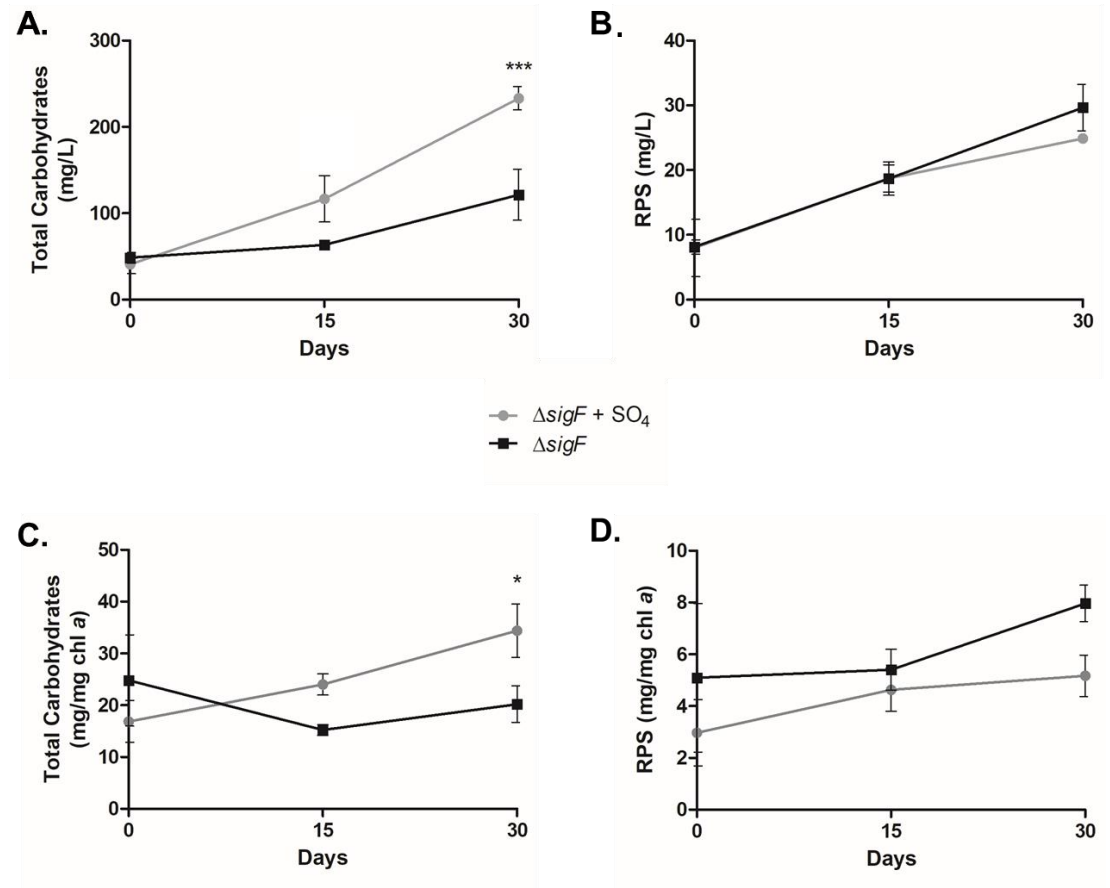

Figure 6. Total carbohydrates and released polysaccharides (RPS) of *Synechocystis* PCC 6803 Δ*sigF* cultures grown in BG11 medium (Δ*sigF*) and in BG11 medium supplemented with sulfate - 1 g L^{-1} $CaSO_4 \cdot 2H_2O$ (Δ*sigF* +SO_4). Total carbohydrates and RPS production were quantified by phenol-sulfuric method and the values are expressed as mg of carbohydrates per liter (L) of culture (A and B, respectively) or mg of carbohydrates per mg of chl *a* (C and D, respectively) (for more details, see *Materials and Methods* section). Experiments were made in triplicate and are represented as mean ± STD (* $p \leq 0.05$, *** $p \leq 0.001$).

In addition, the productions of EPS by *Synechocystis* Δ*sigF* cultures grown with sulfate surplus and in standard condition media were monitored through optical microscopy with Alcian Blue staining, a specific dye for acidic polysaccharides. Once again, no significant differences could be visualized between the RPS produced by Δ*sigF* cells grown with sulfate surplus compared to RPS produced by Δ*sigF* cells grown in BG11 (Fig. 7). This observation, together with the higher content of total carbohydrates, suggests that cultures grown in sulfate-enriched medium can accumulate carbon intracellularly, for example as glycogen or PHB. In fact, another *Synechocystis* sp. PCC 6803 mutant (*slr0977, kpsM*) that showed a similar content of total carbohydrates compared to the wild-type strain, but a significant reduction of the amount of RPS and capsular polysaccharides, displayed an increase of PHB accumulation (Santos et al. 2021). However, further analyses are necessary to determine if indeed any storage compound is significantly increased in Δ*sigF* cultures with sulfate surplus.

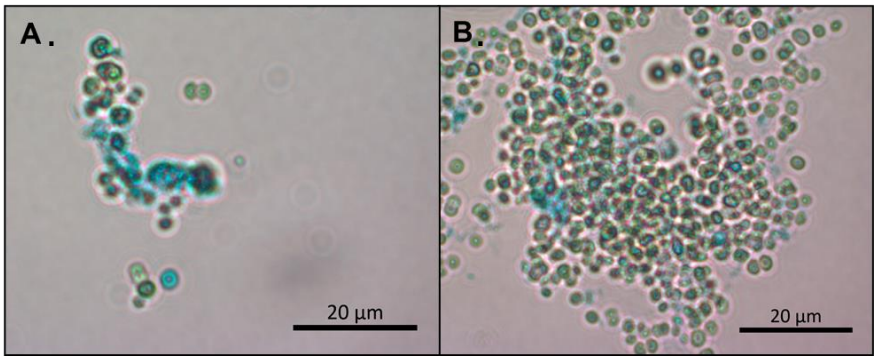

Figure 7. Light micrographs of *Synechocystis* PCC 6803 Δ*sigF* cultures grown in BG11 medium (A) and BG11 medium enriched with sulfate (B) and stained with Alcian Blue highlighting the production of extracellular polysaccharides –EPS (for more details, see *Materials and Methods* section). Scale bar = 20 µm.

After isolate the two Δ*sigF* polymer variants (unmodified Δ*sigF* polymer and Δ*sigF* polymer from cultures with sulfate surplus), their sulfate content was measured using the Sulfate Assay kit. However, the presence of dark precipitates after polymers hydrolysis hindered a feasible quantification of the sulfate (*data not shown*). Therefore, another assay is necessary to be performed in order to correctly determine the sulfate content of the different Δ*sigF* polymer variants, namely ion-exchange chromatography. Due to the laboratory access restrictions this part of the work was not pursued.

3. Manipulation of the polymer peptide content

The peptide content of the unmodified Δ*sigF* polymer and Δ*sigF* polymer from cultures with sulfate surplus, was also manipulated to understand its impact on the antitumor activity. For that, the peptide fraction of these polymer variants was reduced using trichloroacetic acid (TCA) precipitation. After this treatment, the peptide fraction was reduced by approximately 40% in both polymer variants (Fig. 8), with a loss of only ~5% of the initial amount of polymer. These values are in agreement with the efficiency of the peptide precipitation previously reported for other cyanobacterial polymers (Chirasuwan et al. 2007). Moreover, it was verified that the percentage of peptide content of the Δ*sigF* polymer variants from cultures grown in BG11 medium was significatively higher than Δ*sigF* polymer variants from cultures with sulfate surplus (~3 fold) (Fig. 8). This suggests a possible modification regarding the functional groups of the polymer isolated from the Δ*sigF* cells grown with sulfate surplus.

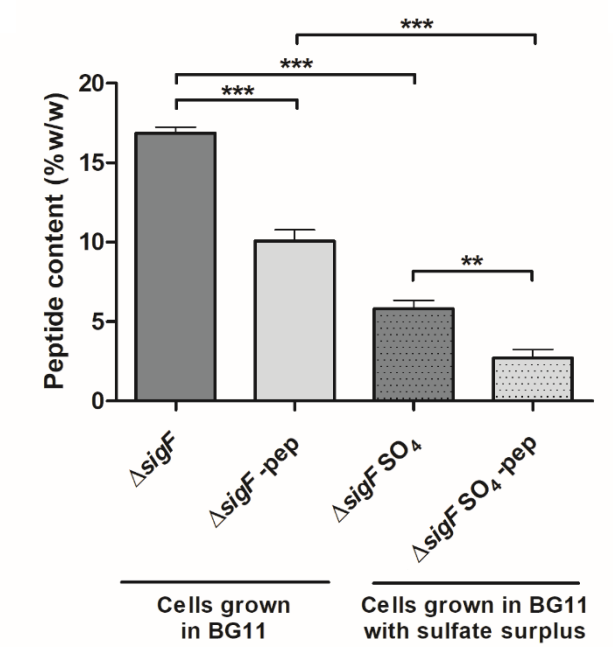

Figure 8. Determination of the peptide content of Δ*sigF* polymer variants from cells grown in BG11 medium or BG11 medium with sulfate surplus before (Δ*sigF* and Δ*sigF* SO_4, respectively) and after trichloroacetic acid (TCA) precipitation (Δ*sigF* -pep and Δ*sigF* SO_4 -pep, respectively) (for more details, see *Materials and Methods* section). Peptide content was quantified by the Lowry method, using bovine serum albumin (BSA) as standard. Experiments were made in triplicates (** $p \leq 0.01$; *** $p \leq 0.001$).

4. Determination of half maximal inhibitory concentration (IC_{50}) for the evaluation of the potential antitumor activity *in vitro*

Preliminary unpublished studies from the research group showed that with the Δ*sigF* polymer with reduced peptide (Δ*sigF* -pep) content had stronger antitumor activity than the unmodified Δ*sigF* polymer towards the human melanoma cell line (Mewo). In addition, the Δ*sigF* polymer from cultures with sulfate surplus did not show significant differences (Silva 2020). Therefore, due to the time and technical constraints mentioned in the previous section we pursued this line of work with the Δ*sigF* -pep by determining its half maximal inhibitory concentration (IC_{50}). For this purpose, a dose-response curve analysis towards Mewo cells was carried out using increasing concentrations (0.0875, 0.175, 0.35, 0.7 and 1.0 mg mL^{-1}). As expected, after 48 h of treatment, a decrease in cell viability was observed with the increase in the polymer concentration. Cell viability was decreased in 50% at the concentration of approximately 0.58 mg mL^{-1} of the Δ*sigF* -pep (Fig. 9), which was lower than the concentration previously determined of the Δ*sigF* polymer (≥0.7 mg mL^{-1}) (Flores, unpublished results).

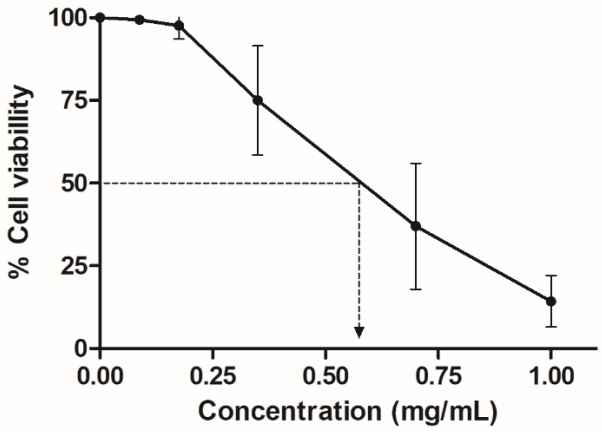

Figure 9. Effect of the *Synechocystis* Δ*sigF* polymer with reduced peptide content on the viability of human melanoma (Mewo) cells, analyzed using the PrestoBlue™ viability assay. The Δ*sigF* polymer was obtained from cultures grown in BG11 medium. The peptide fraction of the polymer was reduced by performing the trichloroacetic acid (TCA) treatment protocol (for more details see *Materials and Methods* section). Cells were treated with different concentrations of the polymer for 48 h. Cells treated with polymer vehicle were also used as controls showing no differences to Blank (*data not shown*). Results are express in relation to Blank and are represented as mean ± STD of four independent experiments.

5. Identification of functional groups in the polymer variants (by Fourier transformed infrared spectroscopy - FTIR)

The functional groups of the unmodified Δ*sigF* polymer (Δ*sigF*) and the Δ*sigF* polymer with reduced peptide content (Δ*sigF* -pep) were identified through Fourier transformed infrared (FTIR) spectroscopy (Fig. 10). The polysaccharide backbone of both analyzed polymers was confirmed, with the band at 1078.07 cm^{-1} and 1041.47 cm^{-1}, which indicate the vibration absorptions of C-O-C ring of polysaccharides, in the Δ*sigF* polymer and the Δ*sigF* -pep, respectively (Fernando et al. 2017, Li et al. 2019). Moreover, the bands ranging from 2921.17 to 2851.12 cm^{-1} are also characteristic peaks of polysaccharides. The FTIR spectrum of the unmodified Δ*sigF* polymer was in agreement with the previously reported in Flores et al. (2019), and reveal a similar pattern regarding the characteristic peaks of polysaccharides for the Δ*sigF* -pep polymer. However, and as expected, some differences were detected corresponding to the amides and amines groups. The major difference in the spectra was around 1535-1550 cm^{-1} that is assigned to the N-H bending related to amide II (Yee et al. 2004). Moreover, the bands at 832.70 $cm^{-1}$and 679.45 cm^{-1}, which are related to the primary amine NH_2 wagging and twisting bands, and secondary amide N-H wagging, respectively (Stuart 2004), were only observed in the Δ*sigF* polymer (Fig. 10A). These results corroborated the efficiency of TCA treatment on Δ*sigF* polymer, since the major differences detected were regarding the functional groups related to the peptides, and corroborate the direct link to the stronger antitumor activity towards the Mewo cell line exhibited by the Δ*sigF* -pep compared to the unmodified Δ*sigF* polymer.

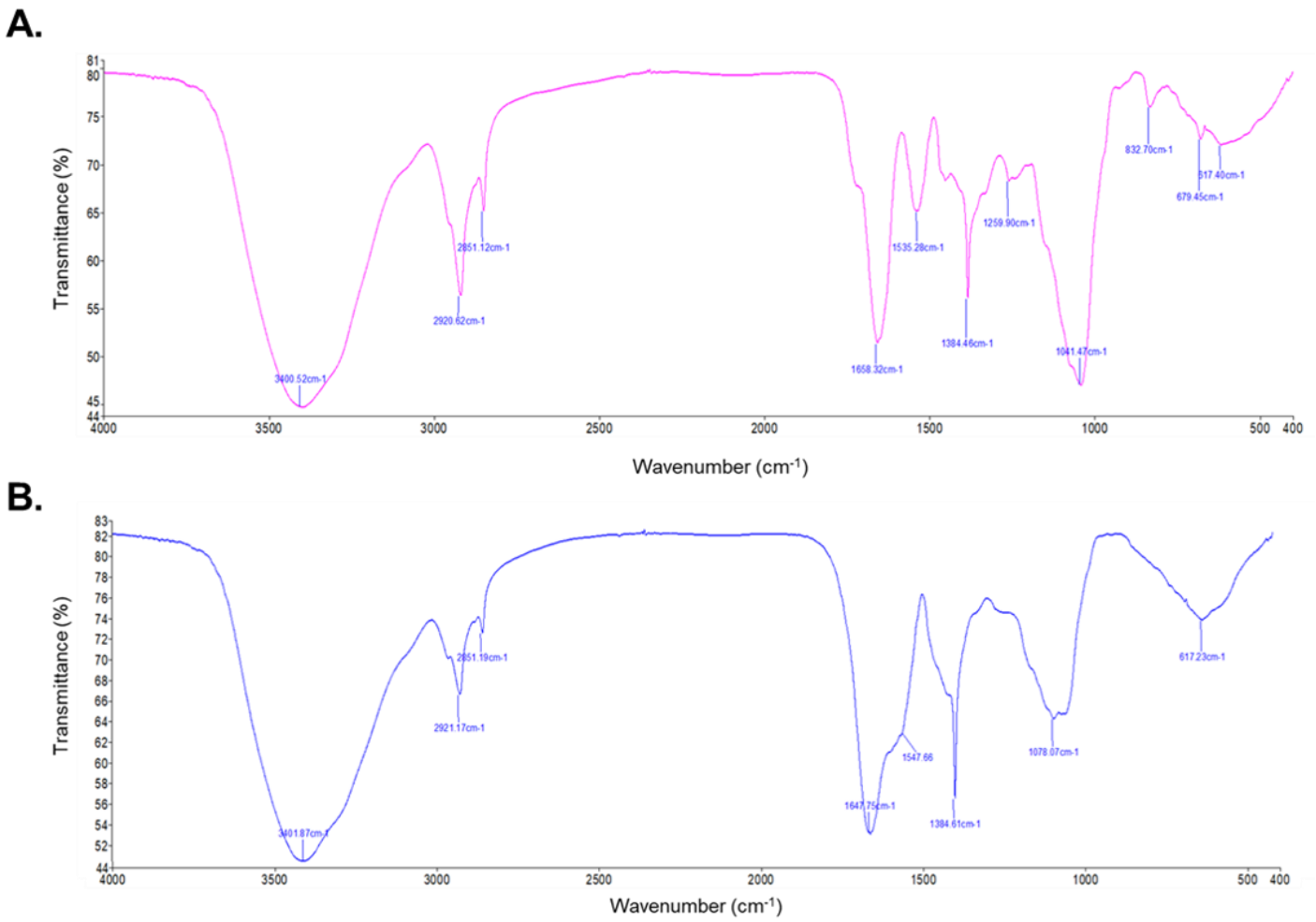

Figure 10. Fourier transformed infrared (FTIR) spectra of the Δ*sigF* polymer (A) and Δ*sigF* polymer with reduced peptide content (B). Values indicate the major absorptions bands and the corresponding wavenumbers. Experiments were made in triplicate and a representative spectrum is shown.

6. *In vivo* assessment of the polymer antitumor activity (chick embryo chorioallantoic membrane - CAM assay)

The chick embryo chorioallantoic membrane (CAM) assay model is a low-cost, reproducible, and reliable preclinical cancer model that allows investigation of tumor growth and angiogenesis *in vivo*, being often used to evaluate potential anticancer drugs (Ferreira et al. 2016). Therefore, and following the *in vitro* results showing that the variant of Δ*sigF* polymer with reduced peptide content (Δ*sigF* -pep) had stronger antitumor potential, a CAM assay was performed. To study the effect of the Δ*sigF* -pep variant and compared to the unmodified Δ*sigF* polymer, Mewo cells were resuspended in the polymer variants at a concentration of 0.7 mg mL^{-1} in DMEM (polymer vehicle) and matrigel, and then inoculated in the CAM model. As controls, cells resuspended in DMEM and matrigel were used. At the end of the experiment, CAMs bearing tumors were fixed, excised from the embryo and photographed *ex ovo* (Fig. 11A). This is the first time that the Mewo cell line was tested by the *in vivo* CAM assays scientific platform at i3S-Institute for Research and Innovation in Health (Univ. Porto). Our results showed that Mewo cells were successfully xenografted in the CAM, presenting a mortality rate comparable with that of untreated/naive embryos (below 10%).

The size of melanoma tumors developed and the angiogenic response was evaluated 4 days after inoculation of Mewo cell line. The results obtained showed that the tumors size decreased significantly independently of the Δ*sigF* polymer variant utilized for the treatment compared to the controls (Fig. 11B). In contrast, no significant differences were observed regarding the number of novel radial blood vessels formed in the tumors treated with the polymer variants solutions or DMEM (Fig. 11C).

It is interesting to notice that, in the *in vitro* assays, the Δ*sigF* -pep polymer showed stronger antitumor activity compared to the unmodified polymer while in our *in vivo* experiment no significant differences were observed regarding the effect of the two polymer variants (using this specific concentration). However, further studies are required, e.g., a detailed histological analysis to assess whether the effect of the polymer variants were distinct at tumor cell morphology.

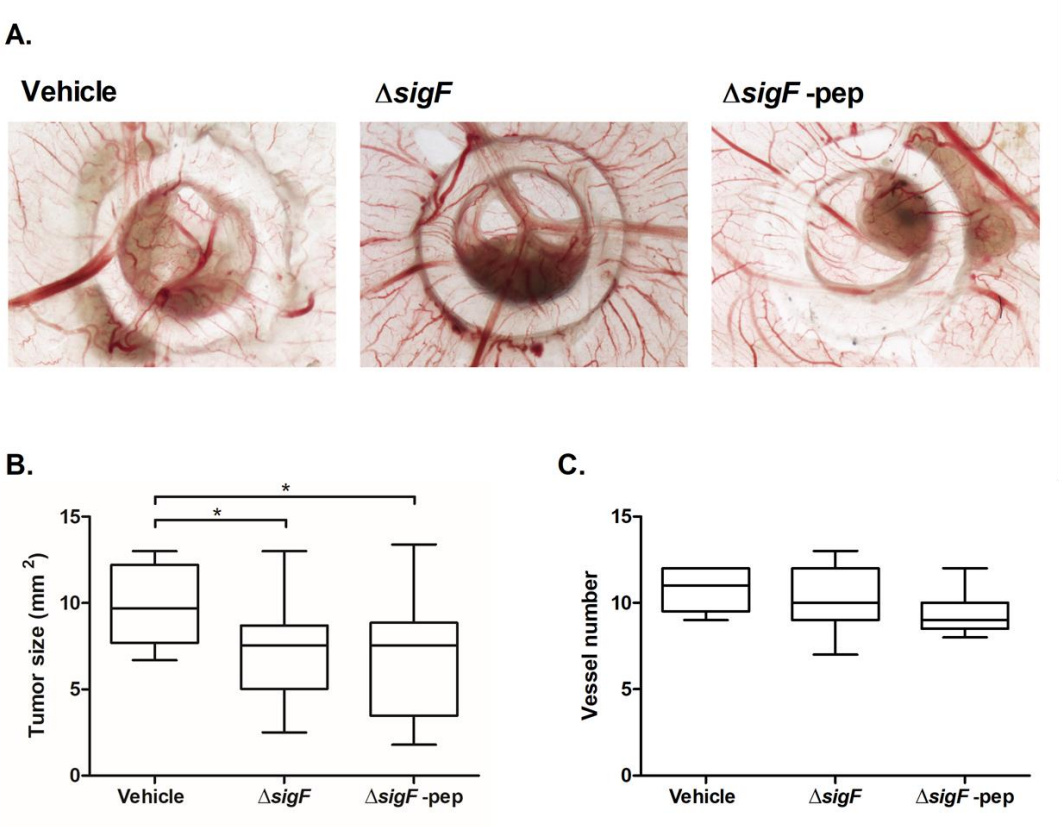

Figure 11. Representative microphotographs of the chick embryo chorioallantoic membrane (CAM) xenografts (A), and evaluation of tumor size in mm^2 (B) and angiogenic response as number of newly formed vessels (C), obtained 4 days after inoculation of Mewo cell line untreated (polymers vehicle) and treated with 0.7 mg mL^{-1} of Δ*sigF* polymer (Δ*sigF*) or Δ*sigF* polymer with reduced peptide content (Δ*sigF* -pep), into the CAM (for more details see *Materials and Methods* section). CAM tumor size was obtained after inoculation of Mewo cells resuspended with vehicle (n=13 eggs), Δ*sigF* (n=18 eggs) or Δ*sigF* -pep (n=17 eggs). Number of newly formed vessels was evaluated after inoculation of Mewo cells resuspended with vehicle (n=9), Δ*sigF* (n=19 eggs) or Δ*sigF* -pep (n=13 eggs). Results are represented as mean ± STD (* $p \leq 0.05$).

Conclusions and future perspectives

Overall, this work contributes to a better understanding of certain polymer features to its antitumor activity. *In vitro*, the Δ*sigF* polymer with reduced peptide content showed strong antitumor activity towards the melanoma cell line (Mewo) compared to the unmodified Δ*sigF* polymer. In the chick embryo chorioallantoic membrane (CAM) assay the two polymer variants reduced tumor growth, validating the polymer bioactivity *in vivo*. However, no significant differences were observed for the two polymer variants, but further studies, e.g. histological analysis, are still required. Moreover, other promising polymer properties could be manipulated (e.g. amino sugars and uronic acids) and their antitumor activity could be tested *in vitro* towards different cell lines, and *in vivo*, using CAM or animal models (e.g. mice).

In summary, this work demonstrates that *Synechocystis* Δ*sigF* polymer/ polymer variants are a potential platform for the study and/or development of a tumor treatment based on cyanobacterial EPS.

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