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Review

Mini-Review: Potential of Diatom-Derived Silica for Biomedical Applications

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Abstract: Diatoms are unicellular eukaryotic microalgae widely distributed in aquatic environments, possessing a porous silica cell wall known as frustule. Diatom frustules are considered as a sustainable source for several industrial applications because of their high biocompatibility and the easiness of surface functionalisation, which make frustules suitable for regenerative medicine and as drug carriers. Frustules are made of hydrated silica, and can be extracted and purified both from living and fossil diatoms using acid treatments or high temperatures. Biosilica frustules have proved to be suitable for biomedical applications, but, unfortunately, they are not officially recognised as safe by governmental food and medical agencies yet. In the present review, we highlight the frustule formation process, the most common purification techniques, as well as advantages and bottlenecks related to the employment of diatom-derived silica for medical purposes, suggesting possible solutions for a large-scale biosilica production.

Keywords: biosilica; diatom frustule; sustainable production; drug delivery

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1. Introduction

Diatoms are an extremely diverse group of algae, comprising more than 100,000 different species [1]. They are able to colonise a large plethora of aquatic environments, and play a significant role on a global scale in the biogeochemical cycles of carbon and silicon in the water column. Two diatom species, *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, have been employed as model species for studies of gene expression and regulation, since they were the first species for which the whole genome was fully sequenced [2,3]. Subsequently, genomes have been sequenced from a number of diatoms possessing specific metabolic or physiological features, such as oleaginous (*Fistulifera solaris*), psicrophylic (*Fragilariopsis cylindrus*), araphid (*Synedra acus* subsp. *radians*), oceanic (*Thalassiosira oceanica*), biofilm-forming (*Seminavis robusta*), and heterotrophic (*Nitzschia* sp.) species [4–9]. Apart from their ecological role, diatoms are also suitable for several biotechnological applications. They can be cultured in the laboratory under sterile conditions and controlled temperatures, light irradiance and nutrient concentrations in order to achieve faster growth rates and to promote the accumulation of specialty products. Diatoms have been employed during the last decades for the production of metabolites exhibiting different biological activities and used as sources for cosmetic ingredients [10], food or feed supplements [11–13], fertilizers [14], and sorbents or accumulators for the bioremediation of aquatic environments [15,16]. Microalgae other than diatoms, especially freshwater green algae, also exhibit a great potential in one or more of the abovementioned fields of research.

The true distinctive feature that makes diatoms more suitable than other taxa for biotechnological purposes, is the high proportion of amorphous silica within their cell wall. This natural source of silicon has already shown several advantages, such as its high surface area and biocompatibility, and can be employed for various research fields, especially for

biomedical applications after in vitro or in vivo treatments [17]. Diatom-derived silica is also available in huge amounts in aquatic benthic environments, as a consequence of the sedimentation of dead diatom cells.

Currently, diatom biosilica is considered as a suitable biomaterial for metal removal from aquatic environments, as a catalyst support, in optical devices, as a microsensor, and other kinds of applications [18,19]. Since its presence on the market as a device for aquatic remediation and as food-grade products is a pledge of its effectiveness in these fields, the present review is mainly focused on evaluating the potential of diatom biosilica for biomedical applications.

Diatom biosilica is actually exploited, indeed, for its potential as a drug carrier [20] and as a scaffold for bone tissue regeneration [21]. Biosilica-based processes can be considered as low-cost and environmentally friendly alternatives to processes based on artificial structures. While the production of synthetic materials requires the implementation of specific protocols, biosilica carries the advantage of triggering natural and sophisticated structure formation. For example, the employment of diatom-derived biosilica for the development of optical sensors may turn out to be, in the future, more attractive than using synthetic crystals, since it allows control and manipulation of light in a cost-effective way [22]. Biotemplated-based silica can be synthesized by rapid environmentally sustainable methods (solvent-free procedures), thus avoiding the use of hazardous chemicals, and allowing a good control of condensation rates [23].

In the last years, the effectiveness of living or fossil diatom-derived silica for biomedical applications (drug loadings, bone tissue regeneration) has been largely investigated by various research groups, and at least four recent reviews clearly summarize the most relevant studies [18,24–26]. In the present review, we pinpoint the major advantages and bottlenecks related to the employment of diatom silica sources. To this aim, we list examples of diatom-based systems that revealed satisfactory results in the laboratory and might be suitable for scale-up in industrial applications, and the few drawbacks that hinder the use of diatom-derived biosilica as a medical device. We critically compared the effectiveness of diatoms silica sources for drug delivery with respect to other biological and non-biological sources. This review also describes the process of frustule formation, the main techniques of silicon purification, and possible solutions to pave the way to silicon production on an industrial scale.

2. Silicon Capture from the Environment, Transport and Storage, Frustule Formation

In contrast with nitrogen and phosphorus metabolisms, silicon uptake in diatoms is linked with aerobic respiration rather than being strictly related to photosynthesis [27]. Silicon is mainly found in aquatic environments as $Si(OH)_4$, and is usually transformed into solid SiO_2 or other siliceous composites in the presence of organic substances, through condensation reactions [28]. It can enter cells by diffusion across their membranes; nevertheless, at very low concentrations of silicic acid in the surrounding environment, the cells activate silicon transporters that facilitate the uptake [29]. The genes coding for silicic acid transporters (SITs) were isolated and characterized from the marine diatom *Cylindrotheca fusiformis* several years ago [30,31] and, recently, in the freshwater species *Synedra ulna* subsp. *danica* [32]. The SITs specifically transport silicic and germanic acids through the lipid bilayer [33]. Studies performed on *C. fusiformis* revealed that ten transmembrane segments allow the passage of silicic acid through the lipid bilayer membrane, and chemical recognition is likely based on amino acids (Figure 1). The extent of frustule silicification depends on the rate of silicon uptake that is driven, in turn, by both the availability of the substrate and the expression levels of the SIT genes [30]. Five distinct clades have been identified for SIT genes in diatoms. The presence of genes from distinct clades within the same diatom species is likely to reflect different responses to changes in silicon availability and environmental conditions [34].

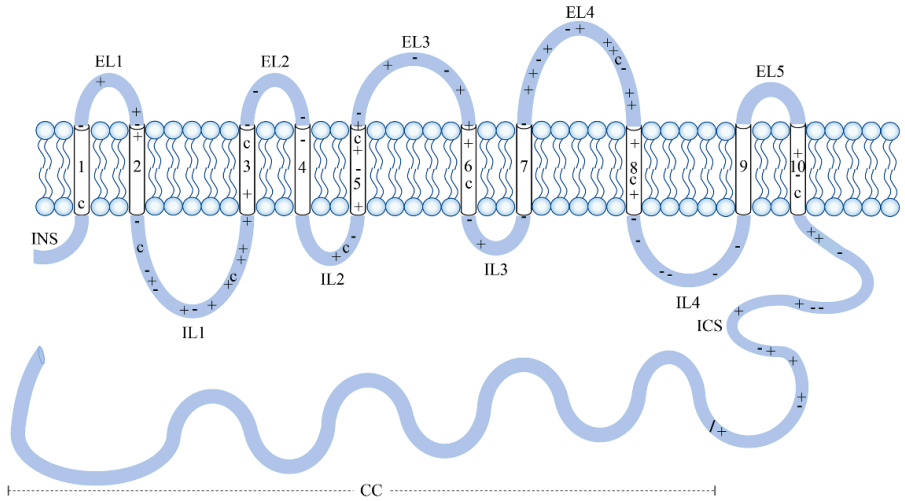

Figure 1. Silicic acid transporters (SITs) in *Cylindrotheca fusiformis*. SITs contain 10 transmembrane segments (white cylinders), which allow the passage of silicic acid through the lipid bilayer membrane; an intracellular amino-terminal segment (INS); and an intracellular carboxy segment (ICS) connected to a coiled-coil motif (CC), which may play a role in the interactions with other proteins. Pluses (+) and minuses (-) indicate the position of positively and negatively charged amino acids, respectively. A major role in silicon uptake is likely played by cysteine residues (C) because of their sulfhydryl blocking agents. Figure redrawn from [31].

Intracellular pools of silicic acid can reach concentrations well beyond the saturation limit (2 mM), and this is likely due to complexation by organic compounds that prevents polymerization. The extent of the internal pools is both species-specific [35] and dependent upon environmental conditions [36]. When silicon deposition and uptake are imbalanced and internal pool tends to increase, a concentration gradient determines the efflux of the "unbound" silicon fraction outside the cell membrane [37].

The diatom cell wall has a Petri dish-like structure, with an upper part, known as epitheca, overlapping the smaller lower part, named hypotheca (Figure 2). Both epitheca and hypotheca consist, in turn, of valves and siliceous girdle bands, which confer an ordered structure to the frustule. Diatom frustule is a composite made of biogenic silica, carbohydrates and glycoproteins [31].

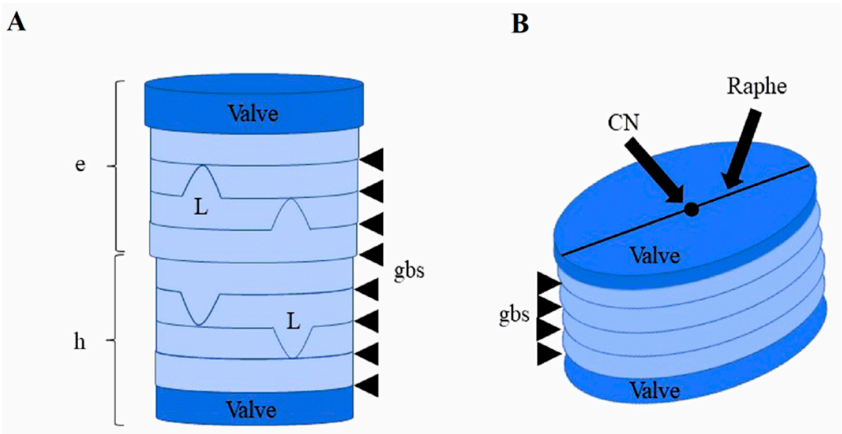

Figure 2. Schematic representation of the cell wall structure of a centric diatom. (**A**) Dark-blue disks represent valves. The upper and larger part is the epytheca (e), the lower and narrower one is the hypotheca (h). Girdle bands (gbs) are indicated by arrows. Ligulas (L) are the bell-shaped structures within girdle bands. (**B**) Diagram of a pennate diatom showing the central nodule (CN) and the raphe on the upper valve. Figure from [38].

Frustule formation occurs through the polymerization of silicic acid in specific compartments, namely, the silica deposition vesicles (SDV), associated to the membrane silicalemma [39,40]. Silicalemma contributes to silicification through both the regulation and recognition of membrane-associated compounds, as well as the formation of a suitable microenvironment for polymerization.

SDVs in diatoms are formed inside the plasma membrane during cell division [41]. During valve formation, the SDVs rapidly expand and their movement is driven by the cytoskeleton [42].

The following several frustule-associated proteins are involved in the formation of the diatom cell wall: frustulins, pleuralins, cingulins, silacidins and silaffins. Aside from proteins, long-chain polyamines, which are constituents of diatom biosilica, are likely involved in silica biogenesis [43].

The following three main levels of cell wall structure organization have been found: (1) microscale, the largest one that determines the outline shape of the valve or girdle band; (2) the mesoscale, at which organized substructures are formed within the SDV; and (3) the nanoscale, which comprises the first products of polymerization and generates different frustule structures/textures of nanometric dimensions ([38] and references therein).

3. Diatom Biosilica Sources

Diatom-derived silica can be obtained either from living cultures or fossil diatoms (diatomite, e.g., chalky deposits of skeletal remains). The energy required for diatom growth is sustained by either led-based (i.e., low energy demanding) artificial light or sunlight. Furthermore, the nutrients required for algal growth, such as nitrates, phosphates, silicates, vitamins, and some trace elements, can be purchased for a relatively cheap price or even obtained from wastewaters. To avoid both the costs of artificial illumination and the seasonal variability of sunlight, cells can also be grown heterotrophically [44–47], although organic substrates are to be supplied in this case. However, only a small number of species are able to grow in the dark [48,49], and organic compounds can promote bacterial growth leading to culture contaminations and to a decrease in cell growth. Biosilica is obtained after cell dewatering (i.e., centrifugation or filtration of the whole culture), followed by a purification process that is usually based on treatments with strong acids and/or high temperatures (see below). Besides, the limited motility of diatoms (due to the lack of flagella) and the "heavy" cell wall (due to the presence of a high silicon amount) enhance the spontaneous sinking of cells, limiting the volume to harvest and, thus, costs of biomass collection.

Diatoms generally exhibit fast growth rates and high lipid and biomass productivities, [50] which can be further enhanced by tuning growth conditions [51,52], making diatoms promising candidates for mass culturing. However, to the best of our knowledge, no diatom-based industrial plants (i.e., indoor or outdoor systems of algal culturing) are focusing on biosilica production as their main activity. Follow-up studies are thus required to lay the foundations for the industrial production of silica-based biomaterials.

The most abundant source of biosilica that does not foresee the induction of living cultures is diatomite, which can be easily crushed into a fine powder to become a marketable product, namely, diatomaceous earth (DE). Diatomite is made of frustules of dead diatom cells, usually found in benthic environments. The harvesting of fossil frustules, which are naturally present in benthic environments, is cost-effective and makes diatomite a promising starter for the industrial production of biosilica. However, the composition of DE is variable and the purity is often lower than that of living culture-derived frustules. The quality and abundance of these impurities vary upon environmental and aging conditions [18]. DE, generally made of ca. 80–90% of silicon and of clay minerals [53], is used as a raw material for different kinds of applications, such as agricultural fertiliser, sorbent for pollutants, and filler in plastics and paints to improve the strength of construction materials. In addition, DE is also employed to filter impurities and as an abrasive agent in cleaning and polishing products.

4. Frustule Cleaning/Purification: Main Techniques and Technical Issues

Frustules can be thus purified from both living culture-derived algal biomass and diatomite stocks. The impurities of diatom frustules mainly consist of organic matters adhered to their surface [54]. In the case of diatomite samples, impurities are present in larger amounts, and can vary in relation to the local environment and aging conditions of these natural stocks [18]. Diatomite impurities typically contain also clay and metallic oxides, such as aluminium and ferric oxides [55]. Before cleaning procedures, diatomite particles usually undergo a first step of pulverization, in which micrometric powder is grinded to nanoparticles by mechanical crushing and sonication. However, apart from a few exceptions, most studies report purification protocols based on raw material derived from living cultures rather than diatomite, which is currently the only diatomic silica-based marketable product.

Organic impurities can be removed from the silica frustule by either a chemical pre-treatment with acids or other oxidative agents, or by exposing the frustules to high temperatures. Some studies, aimed at assessing the efficacy of preliminary hydrochloric acid treatments for organic mass removal, showed that acid concentration greatly influenced both the removal rate of impurities and the state of preservation of the frustule shape, with strong acidic pre-treatments causing frustule erosion [56]. Potassium permanganate can be also used to pre-treat frustules for organic compound removal [57,58]. However, this procedure is essentially limited to remove impurities outside the frustule, and pre-treatments with acidic solutions are usually applied (even if they are not mandatory) when purification protocols do not foresee acid-based cleaning procedures, such as baking-based purifications [59]. Some preliminary oxidations with acid solutions do not exclude the employment of both acids and high temperatures. Treatment of diatom frustules with sodium permanganate and oxalic acid, for example, is followed by perchloric acid treatments at 100 °C [57].

Baking (i.e., strong heating of silica cell walls) of diatom frustules at 400–800 °C is the simplest and least expensive method to remove organic components. However, high-temperature treatments can alter diatom architecture and pore size [60]. Oxygen plasma etching, a procedure consisting of the removal of impurities using ionised gases, was found to be effective to preserve the frustule structure, with a negligible loss of material and without shape alterations [61,62].

The most commonly used procedure for the removal of organic matter and the purification of diatom biosilica is, however, an oxidative washing treatment. Some protocols require the use of 30% [54,63–67] or 15% [68] hydrogen peroxide solutions.

The most common washing solvents used in acid-based treatments of diatom frustules are sulphuric [69,70] and nitric [68,71] acids. Sulphuric acid treatment is rapid (10–30 min) and revealed successful even on small amounts of biosilica [55]. Despite the rapidity of this strong acid-based method, cleaning procedures are time-consuming, since several washes with distilled/deionised water are required for a complete acid removal. However, the effect of acid strength needs to be evaluated in each case, since silica nanostructures can be damaged by the action of acids. For example, frustules from poorly silicified diatom species can be dissolved in strong acid cleaning solutions [70].

To improve the efficiency of biosilica purification, Wang and co-workers [72] set up a vacuum cleaning method in which all the cleaning steps, which are cell extraction, acid treatment and washing, are carried out on polytetrafluoroethylene (PTFE) filter cloths, thus decreasing the processing time. This allows the recycling of the sulphuric acid used for cleaning, decreasing the amount of both the reagent needed for purification and the liquid wastes. The main drawback of the vacuum cleaning method is that it depends on the mechanical properties of the raw material, and cannot be applied on poorly silicified diatoms.

Some purification methods combine the use of both sulphuric acid and hydrogen peroxide in a strong oxidizing agent (2 M H_2SO_4, 10% H_2O_2) called Piranha solution [26,73]. The purification process is relatively fast, while post-treatment washes can be time-consuming.

The removal of Piranha solution requires, indeed, an overnight treatment with HCl (5 M, 80 °C) and two further washes with distilled water to eliminate the HCl residuals [20]. The main treatments for frustule separations, the tested diatom silica sources, and the main bottlenecks of each cleaning technique are summarized in Table 1.

Table 1. Pre-treatments and treatments for diatom frustule cleaning and their main advantages and drawbacks.

	Treatment	Principle for Organic Matter Removal	Diatom Species	Diatom Silica Source	Advantages	Drawbacks	Reference(s)
Pre-treatments	HCl	oxidizing washing	*Nitzschia closterium*, *Thalassiosira* sp.	freeze-dried samples	high purity of frustules	possible frustule erosion depending on acid strength	[56]
	$KMnO_4$ + $C_2H_2O_4$	oxidizing washing	*Fragilariopsis cylindrus*, *Fragilariopsis kerguelensis*, *Pseudonitzschia seriata*, *Thalassiosira nordenskioeldii*, *Thalassiosira aestivalis*, *Thalassiosira pseudonana*, *Thalassiosira weissflogii*	wet pellets washed with sodium lauryl sulfate	no frustule erosion	removal of the only external organic matter	[57,58]
Treatments	baking	high temperature	*Navicula* sp.	APS-fuctionalised diatoms on a mika surface	reduction in hazardous chemicals	possible alterations of pore size, possible post-treatments with acid solutions	[60]
	low-temperature plasma ashing	ionised gas	*Navicula*, *Amphora*, *Cocconeis*, *Planothidium* spp.	desalted drops of cultures, freeze-dried samples	no frustule dissolution	unsuitable for saltwater species, expensive, post-treatments with hazardous chemicals	[61,62]
	H_2O_2	oxidation	DE, *Ni tzschia frustulum*, *Pinnularia* and *Coscinodiscus* spp., *Thalassiosira pseudonana*, *Cylindrotheca closterium*	desalted and freeze-dried cultures, diatom composites	less dangerous than strong acids	long incubation, high-temperature post-treatments needful to increase efficiency	[54,62–67]
	H_2SO_4	strong oxidation	*Thalassiosira rotula*, *Coscinodiscus wailesii*	living cultures	high efficiency in organic matter removal	hazardous chemicals, dissolution of thin frustules, time-consuming post treatments	[69,70]
	H_2SO_4 + PTFE filters	strong oxidation under vacuum	*Nitzschia*, *Ditylum*, *Skeletonema*, *Coscinodiscus*	living cultures on a filter cloth	reduced acid amounts	unsuitable for thin frustules	[72]
	HNO_3	strong oxidation	*Pinnularia* sp., *Coscinodiscus concinnus*	harvested cells	high efficiency in organic matter removal	high-temperature treatments needful to increase efficiency	[68,71]
	Piranha solution (H_2SO_4 + H_2O_2)	strong oxidation	*Thalassiosira pseudonana*	PBS-washed cells	high efficiency in organic matter removal	time-consuming post-treatments	[73]

5. Silica for Biomedical Applications: Advantages

The main benefits of biosilica for biomedical purposes are as follows: plasticity of frustules for functionalization, biocompatibility, possibility of genetic transformation of living cultures for protein immobilization, and high availability of silica-derived diatoms.

The biosilica derived from diatoms requires cheap synthesis processes [26], and is also characterised by chemical inertness, low or null toxicity, thermal stability and high availability [18]. Silica has been widely investigated in drug delivery systems because of its high robustness and versatility compared to other materials [74], and frustules derived from both living cultures and diatomite particles have successfully been employed as drug carriers [73,75].

5.1. Surface Functionalization for Drug Loading and for Biosensing Chips for Biomedical Applications

Frustule functionalization consists of modifying its surface to enable the formation of stable covalent bonds with proteins or DNA [26,76], by introducing chemically reactive species functioning as cross-linkers. This step is crucial to improve the quality of the resulting material for specific applications. Chemical modification of biosilica can be critical, for example, to regulate the kinetics of drug release, and the high surface-to-volume ratio makes this raw material particularly suitable for drug delivery. Diatom frustules are characterized by precise and species-specific cell morphologies, and both the size and shape can highly differ among distinct diatom taxa. It has been estimated that the surface area ranges between 1.4 and 51 $m^2\ g^{-1}$ [77–80]. The size and the architecture of the pores are likely to influence drug release [75].

Drug release in biosilica-based systems is usually characterized by the following two phases: a first phase of fast release, due to the detachment of drug molecules weakly bound to the frustule surface, and a slow releasing phase, due to drug delivery from the internal pore structure of diatom frustules [81]. Chemical modifications of diatom-derived biosilica allow their use as a carrier of both soluble and insoluble drugs.

The effectiveness of DEs as delivery systems for the drugs gentamicin (soluble) and indomethacin (insoluble) was demonstrated in previous studies [67], in which DE was modified with a self-assembling monolayer (SAM) including organosilanes and phosphonic acids, thus rendering the diatom frustules hydrophilic or hydrophobic, respectively, before drug loading. A sustained release of indomethacin, which has been exploited as a model drug for silica-based devices, was also demonstrated with DE particles functionalised by dopamine-modified iron oxide nanoparticles (DOPA/Fe_3O_4 nanoparticles). Diatom-derived silica was employed, in this case, as a magnetically guided micro-carrier for drug delivery, since dopamine amino groups on the diatom surface allow the attachment of targeting biomolecules [78]. Another kind of functionalization can be obtained by combining the frustule with graphene oxide (GO) sheets through covalent bindings. These nano-hybrid composites are suitable drug microcarriers. GO sheets enhanced, indeed, drug-surface interactions, improving the kinetics of drug release [82].

Silica functionalization was also used to counteract cancer progression, through the delivery of water-insoluble antitumor drugs. A recent study showed that DE particles coated with vitamin B12 allowed better delivery of cisplatin and 5-fluorouracil (5-FU), two anticancer agents effective against colorectal cancer cells [83]. Silicon nanoparticles (SiNPs) were also functionalized with 5-FU and the chemopreventive agent curcumin, and then encapsulated into acid-resistant microspheres to show the effectiveness of oral administration of these chemotherapeutics against colorectal cancer [84].

DE particles were also used as a solid drug-carrier in phospholipid suspensions for new oral formulations of non-anticancer water-insoluble drugs, such as the anticonvulsive carbamazepine [85].

While the abovementioned applications of biosilica were all based on the employment of fossil sources, other studies were focused on culture-derived biosilica. Functionalised frustules of the diatom *Nitzschia palea* have been successfully exploited as carriers for the antibacterial complex tyrosine-Zn(II); zinc ions covalently bounded to the frustule surface showed, indeed, a toxic effect on bacteria, thus reducing their concentration [86]. Esfandyari et al. [87] exploited the potential of *Chaetoceros* sp. frustules to detect circulating tumour cells. Diatoms were magnetized with iron oxide nanoparticles, and then conjugated with the monoclonal antibody Trastuzumab; this system was effective in selectively targeting

and separating breast cancer cells, SKBR3 cells (HER2 positive cells), from HER2-negative cells under a magnetic field. The optical properties of these diatoms allowed to detect this specific binding ability by fluorescence microscopy, thanks to the optical properties of the silica.

Similar studies on antibody-functionalized nanoparticles deriving from living cultures were already performed more than ten years ago, and they exploited the potential of two modified centric diatoms as photoluminescent biosensors. Functionalization of *Coscinodiscus wailesii* frustules was one of the pioneer studies highlighting antigen recognition from antibodies that had been covalently bound to frustules [88]. Gale and co-workers [89] succeeded in transforming *Cyclotella* sp. frustules with the model rabbit IgG antibody, showing a correlation between the photoluminescence associated with the frustule/antibody complex and the antigen (goat anti-rabbit IgG) concentration. The main types of diatom silica functionalization are summarised in Table 2.

Table 2. Sources, type of functionalization and biomedical applications of diatom-derived biosilica.

Diatom Source	Type of Functionalization	Main Application	Aim	Reference(s)
Coscinodiscus wailesii	Silanization and antibody conjugation	Biosensor	Specific recognition antigen–antibody (murine monoclonal antibody)	[88]
Coscinodiscus wailesii	Silanization and antibody conjugation	Biosensor	Tethering and detecting antibodies (mix of normal rabbit serum and purified Ig-Y)	[64]
Cyclotella sp.	Silanization and antibody conjugation	Biosensor	Selective and label-free photoluminescence-based detection of antigen–antibody (IgG-rabbit) complex formation	[89]
Chaetoceros sp.	Iron oxide nanoparticles and antibody conjugation	Biosensor (with magnetic properties)	Selective targeting of SKBR3 cancer cells through the employment of antibody (Trastuzumab) bioconjugation	[87]
Thalassiosira weissflogii	Nitroxide 2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) conjugation	Drug carrier	Ciprofloxacin delivery in fibroblasts and osteoblasts	[90]
Aulacoseira sp.	Silanization, and oligo (ethylene glycol) methacrylate copolymers addition	Drug carrier	Improvement of levofloxacin delivery	[75]
Nitzschia palea	Amino acid (Tyr-Zn^{II}) conjugation	Drug carrier	Inhibition of bacterial growth	[86]
Diatomaceous earth	Silanization and phosphonic acids conjugation—self-assembling monolayer	Drug carrier	Improvement of indomethacin and gentamicin delivery	[67]
Diatomaceous earth	Silanization and phosphonic acids modifications	Drug carrier	Improvement of indomethacin delivery	[91]
DE mineral rocks	Graphene oxide, silanization	Drug carrier	Improvement of indomethacin delivery	[82]
Diatomaceous earth	Dopamine modified iron-oxide nanoparticles (DOPA/Fe_3O_4)	Drug carrier (with magnetic properties)	Improvement of indomethacin delivery	[78]
Diatomaceous earth	vitamin B12 and ruthenium (II) complex	Drug carrier	Improvement of the anticancer tris-tetraethyl [2,2′-bipyridine]-4,4′-diamine–ruthenium (II) complex delivery (tested on HT-29 and MCF-7 cancer cells)	[83]
Calcined diatomite	Silanization and siRNA conjugation	Drug carrier	Vehiculating siRNA into tumour cells to downregulate the expression of cancer-associated genes (tested on murine A20 lymphoma cells)	[92]
Calcined diatomite	Silanization and siRNA conjugation	Drug carrier	Vehiculating siRNA into tumour cells to downregulate the expression of cancer-associated genes (tested on H1355 cancer cells)	[93]

5.2. Biocompatibility

Diatom-derived biosilica has several advantages compared to other porous materials, in terms of high compatibility with biological systems [18,26]. Biocompatibility tests were performed on various tumour cells, and some significant examples are reported below. An ATP-based luminescent assay aimed at detecting the short-time (6–24 h) detrimental effects on cells showed that DE particles had very low toxicity on the following three colon cancer cell lines: Caco-2, HT-29, and HCT-116 [94]. The effect of amino-modified DE nanoparticles on human lung epidermoid carcinoma cells (H1355) was evaluated by the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Different concentrations of diatom particles were tested for 24, 48 and 72 h, and the results showed very low cytotoxicity against the abovementioned tumour cells. This feature made functionalized DE particles useful carriers to transport small interfering ribonucleic acid (SiRNA) inside human lung epidermoid carcinoma cells (H1355), silencing gene expression [93]. Biocompatibility was also assessed on bone cells, such as normal human dermal fibroblasts (NDHS) and Saos-2 osteoblasts, by the functionalization of *Thalassiosira weissflogii* frustules with 3-mercaptopropyl-trimethoxysilane (MPTMS). The mercapto-coated biosilica successfully stimulated the growth of both cell lines, even more than bare cells [90].

The biological compatibility of silica-derived diatoms was also assessed in studies aimed at targeting the antiapoptotic factor B-cell lymphoma/leukemia 2 (Bcl2) with small interfering RNA (siRNA). Specifically, the amino groups of silanized silica particles were complexed with siRNA to downregulate the expression of tumour-associated genes. The target line was the A20 murine lymphoma, and no differences in cytotoxicity between the functionalised frustules and controls (e.g., untreated cells) were observed by applying the following three different methodologies: MTT, Cell-Titer GLO and propidium iodide assays [92].

Biocompatibility between functionalized DE particles and breast cancer cells (lines MCF-7 and MDA-MB-231) has also been proven. In this case, amino-modified particles were further improved by PEGylation (i.e., diatom-coating with polyethylene glycol) and cell-penetrating peptide (CPP) bioconjugation, to promote cell internalization through physical and biological changes in the silicon source. The biological compatibility was also evaluated with a luminescent cell viability assay based on the adenosine triphosphate concentration, and the results showed that the cytotoxicity of biosilica that underwent a double modification with PEG and CCP was lower than that of the bare material, as well as that of diatoms that had been amino-modified only [95].

Most cytotoxicity assays mentioned above were performed on short timescales. The effect of longer exposure times (21 days) was assessed on human embryonic kidney cells (HEK-293) and MDA-MB-231 breast cancer cells exposed to syntherized (e.g., fused at high temperature) diatoms. Biocompatibility was tested through viability assays with the dye Calcein-AM (its fluorescence intensity depends on the activity of cellular esterases, and thus of viable cells), and the results confirmed that natural silicon is not toxic. This suggested that fused diatom frustules could be a suitable alternative for synthetic bone graft substitutes [96]. In order to foresee the effects of long-term exposure of silica-based devices on biological systems, Terracciano and co-workers [97] investigated the in vivo impact of diatomite particles on the model organism *Hydra vulgaris*. Untreated specimens and animals exposed to bare frustules and to diatom nanoparticles modified with the cell-penetrating peptide [(aminooxy)acetyl]-Lys-(Arg)9 (to enhance cellular uptake) were monitored for 14 days, and no detrimental effects in terms of growth rates and apoptosis were observed in all conditions.

In our opinion, further studies on living organisms are mandatory to definitely ascertain the lack of toxicity of biosilica, especially in the perspective of concrete biomedical applications for drug loading and as scaffolds for bone regeneration.

5.3. Employment of Genetically Engineered Diatom Frustules for Protein Immobilization

Diatom particles can be considered as useful scaffolds for enzyme immobilization that could enhance protein properties. Genetic engineering represents a viable alternative to in vitro immobilization systems, as it does not require protein purification and is carried out under physiological conditions [74]. Since silaffins and cingulins are involved in silica condensation becoming part of diatom frustules, the fusion of an exogenous protein to these frustule-associated proteins can result in the strong binding of exogenous proteins to the silica cell wall.

Transformation of diatom genomes with recombinant genes is a useful tool to allow the fusion between enzymes and cell wall proteins. This technology is mentioned in a recent study as living diatom silica immobilization (LiDSI), and has been mostly performed on the model species *T. pseudonana* [98,99]. To our knowledge, the pioneer studies focused on enzymes immobilised on diatom biosilica were aimed at inserting and blocking the bacterial enzyme hydroxylaminobenzene mutase (HabB) on the silaffin tpSil3 of *T. pseudonana* frustule [98]. Aside from the potential of this specific genome modification, this study paved the way for the genetic manipulation of diatom species to enhance protein immobilization on frustules for biomedical purposes.

The genome of *T. pseudonana* has been recently modified with the insertion of exogenous genes encoding the fusion of two enzymes, glucose oxidase and horseradish peroxidase, with cell wall proteins, enabling a regioselective functionalization, and suggesting that silica morphology could influence the effectiveness of the enzymes reactivity [99]. The frustule of this species has been also antibody-functionalised, in order to test its effectiveness in binding large and small antigen molecules [100].

5.4. Availability of Biosilica Feedstocks

In contrast with other synthetic materials, diatom biosilica is already available in huge amounts as diatomite. Moreover, diatom-derived silica feedstock could be easily obtained by culturing these microalgae in open ponds or enclosed systems, and separating them from the organic matter after culture dewatering.

6. Silica for Biomedical Applications: Bottlenecks

Among the weak aspects of the production of biosilica-based devices, it is worth mentioning that diatom frustules require strong treatments that are usually based on toxic and/or dangerous chemicals [26,70,73,101]. Furthermore, the accuracy of the purification protocols should to be the highest possible in case the biosilica-based material is to be used for biomedical purposes. Besides, experiments based on living culture manipulations are often carried out under axenic conditions [87,89], which requires very careful maintenance to keep the strains bacteria-free and, in case of contamination, highly meticulous protocols to remove bacteria need also to be applied [102].

Another drawback of using biosilica for biomedical purposes is the low degradation rate of this material. Although biosilica is known to be less stable than crystalline silica and its dissolution rate can be further enhanced by specific physico-chemical manipulations [103], it can still persist for long periods within organs with limited blood supply [104], which may lead to detrimental health consequences. Some authors do not consider biosilica degradation as a real problem, since the silicic acid (i.e., the main product of silica degradation) is naturally found in human tissues and can be easily excreted from the kidneys [105,106].

However, the employment of diatom-based biosilica for biomedical purposes has not been approved yet by the Food and Drug Administration (FDA), and neither by other safety governmental agencies. The approval of diatom-based biosilica for biomedical purposes is mandatory to follow up all the studies which foresee the development of biosilica-based devices for biomedical purposes. The FDA considers amorphous silica less toxic than crystalline forms and silicates as generally recognized as safe (GRAS) materials [57,107], and established that they can be included in oral delivery ingredients in amounts up to 1500

mg per day [103]. Some diatom-derived sources of silicon can be treated, indeed, to reach the food grade and can be used for nutraceutical purposes. Currently, diatomite of certain "purity levels" is considered as a food additive and is permitted as animal feed (https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=573.340, accessed date: 22 March 2021), as inert carrier or anticaking agent, and as additive for pharmaceutical preparations. Unfortunately, the use of diatomite for biomedical applications has not been approved yet.

The recognition of diatomite as a suitable biomaterial for medical and pharmaceutical purposes by the FDA is, indeed, mandatory to pave the way to industrial applications of diatomite-based materials, which have proven to be successful for drug delivery [74], through genetic engineering [108,109] and for regenerative medicine [26].

7. Future Perspectives

In the present review, we report studies from different research groups aimed at assessing the effectiveness of diatom-derived silica for biomedical applications. Previous studies clearly demonstrated that diatom biosilica could be suitable as a drug carrier, as a scaffold for regenerative medicine, and for in vivo enzyme immobilization. We summarized the main benefits and disadvantages associated with the use of this biomaterial, and, in our opinion, the advantages outweigh the drawbacks. Future research should ascertain long-term compatibility of biosilica with biological systems, and should search for cost-effective and environmentally friendly techniques for biosilica production.

The employment of silica-based diatom cell walls for biotechnological applications implies that naturally abundant fossil sources (e.g., diatomite) can be considered as a viable and cost-efficient alternative to synthetic silica, which requires time-consuming and hazardous methods for its production. Overall, fossil biosilica sources can be used as heavy metal sorbents, food-grade additives, fertilizers or biosensors.

In contrast, living diatom-derived biosilica appears to be more suitable than biosilica of fossil origin for biomedical applications such as drug carriers. Specifically, drugs need to be released from the carrier at constant and known rates, and for this purpose the size, shape, and porosity of biosilica particles should vary as little as possible. Biosilica derived from monospecific cultures has, indeed, a regular and predictable structure compared to fossil sources that typically encompass a given morphological diversity, leading to heterogeneity in the frustule size, shape and porosity. A recent device for biosilica production from living cultures has been patented [110] and paves the way to the exploitation of diatoms.

With respect to other biological silica sources, such as sponges and Radiolaria, diatoms can be easily cultured in the laboratory, and are ubiquitous and highly abundant in the marine environment. Moreover, silica structures from diatoms generally have smaller dimensions than protozoan shells and sponge spicules, which can reach 2 mm in length [74]. The reduced particle size is, for us, advantageous in drug delivery systems to enhance degradability, and for avoiding or limiting pulverization pre-treatments. In our opinion, treatments for the depigmentation and elimination of collagen (in which spicules are enveloped) can render the process of sponge-derived silica even more difficult than the typical techniques for frustule purification [111,112]. Other non-biological silica sources, such as fly ash derived from power plants, may be used in lieu of fossil diatom-derived sources. Similarly to diatomite, this alternative source is disposable and abundant in the natural environment, and its effectiveness as a raw material for carbon sequestration [113] and as a biofertilizer [114] has already been demonstrated. Nevertheless, in our opinion, the high heterogeneity of fly ash (structural features vary according to the power plant and the combustion processes), and the presence of metals and other impurities makes it more suitable for bioremediation and land fertilization purposes, rather than biomedical applications.

To improve the economic viability of massive diatom culturing for biosilica production, other fractions of algal biomass could also be exploited. Diatoms can be used, indeed, as photosynthetic biorefineries [115] for a number of industrial processes, and the exploitation of both the inorganic and organic fractions of the algal biomass can contribute to minimise

waste production. A possible route for the complete exploitation of diatom biomass is shown in Figure 3. The extraction of the biochemical components from microalgal biomass does not affect the structure and the integrity of silica frustules, which can remain unaltered also in the presence of acidic conditions. Massive diatom culturing can lead to the combined production of biosilica for biomedical applications, and highly valuable organic compounds such as PUFAs and carotenoids.

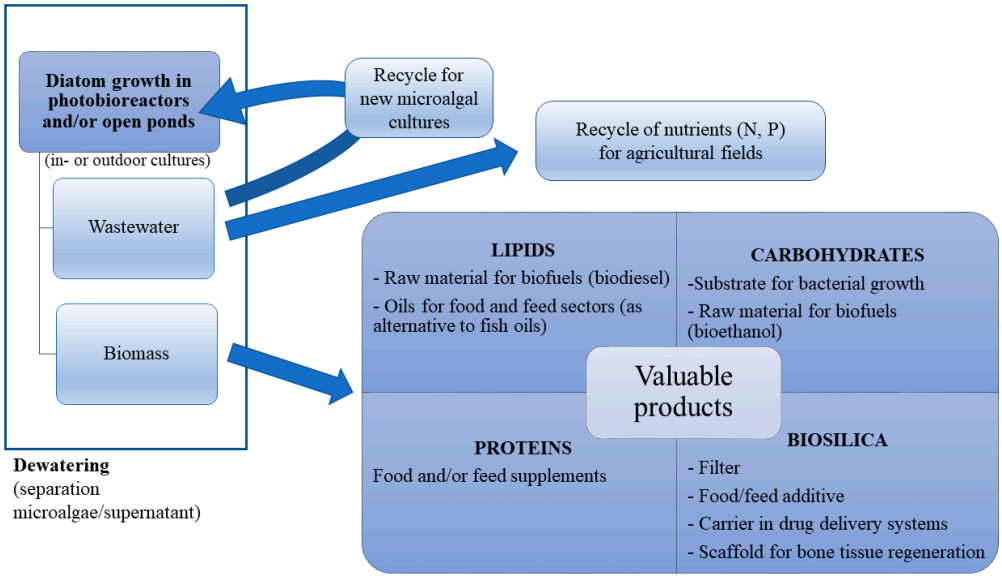

Figure 3. Schematic representation of a hypothetic diatom-based biorefinery for the whole exploitation of microalgal mass. Diatoms are cultured in open ponds or photobioreactors; after harvesting, the remaining water is still rich in nutrients and can be partially reused as medium for new culture inocula or serve as agricultural fertiliser. Four major valuable products, lipids, proteins, carbohydrates, and biosilica can be obtained from the harvested biomass.

In summary, we believe that further studies are required to exclude any acute and long-term toxicity of diatom biosilica—as suggested by Castillo and Vallet-Regi [116]—and this will pave the way for clinical trials of biosilica transplantation. However, assessing the best processes to minimize costs and wastes for the concomitant production of biosilica and highly valuable products is mandatory to lay the foundations for this new industrial application.

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