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Ph.D. Thesis

Biosilica as a New Packing Material for Chromatographic Separations

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1. INTRODUCTION

Chromatography is an extremely important and frequently used technology in analytical chemistry because it is necessary to understand what is inside the sample. Chromatography is a method for separating substances based on how different the affinity for or solubility is in two different phases (the stationary phase, and the mobile phase). The concept of chromatography was first mentioned in the University of Warsaw more than 100 years ago by Tswett as he used a glass tube packed with calcium carbonate and flushed it with a mixture of organic solvents to separate plant pigments [1]. This early type of chromatography is still widely practiced today as flash chromatography mostly by organic chemists. Since performing chromatography in this manner was typical for a very long time, it was referred to as "*normal phase*" chromatography. However, there is still an opportunity for these techniques to develop a new instrument model, chromatographic columns, and chromatographic system technical solutions to satisfy the various new requirements and requests for improvement from other scientific areas as well as an increase in the precision and accuracy of measurement. The "*heart*" of every chromatographic system is the chromatographic column. Of all the chromatographic constituents, its development is most susceptible to alteration to reduce the changes in chromatographic properties between batches that are manufactured consecutively therefore, researchers are continually concentrating their efforts on increasing the effectiveness and longevity of columns [2]. Modern High-Performance Liquid Chromatography (HPLC) column has been categorized into numerous subclasses based on the mode of separation, the polarity of the mobile phase and stationary phase, and the type of intermolecular interaction that dominates the retention process as follows: a) Normal Phase Liquid Chromatography (NPLC) - a chromatographic system in which the stationary phase is more polar than the mobile phase, b) Reverse Phase Liquid Chromatography (RPLC) - a chromatographic system in which the stationary phase polarity lower than that of the eluent used, c) Hydrophilic Interaction Liquid Chromatography (HILIC) a chromatographic migration system which is a combination of the two above-mentioned systems, with a stationary phase used in the normal phase system(polar) and mobile phase used in a reversed-phase system (acetonitrile with water). The most widely used HPLC columns are RP-HPLC columns, which account for more than 90% of all HPLC

separations [3,4]. On one hand, in liquid chromatography, the basic material used from the beginning of chromatographic analysis until the present day is amorphous silica material. This is due to the low costs associated with the high availability of silicon in the natural environment and the presence of superficial silanols, which are essential for liquid chromatographic (LC) stationary phase synthesis and column preparation [5]. They provide the hydrophilic nature of silica, which feature is directly used in NPLC, but they also create the possibility of attaching organic moieties to them. The higher concentration of silanols on the surface makes the silica more hydrophilic in nature. Since the presence of surface silanols affects the selectivity of a stationary phase in all LC modes, it is necessary to know their concentration on the surface. Currently, the silica used for LC column packing is a synthetic silica which allows greater control of the final product. The appropriate selection of synthesis parameters ensures obtaining appropriate particle sizes, their spherical shape, porosity, and the proper concentration of silanols on the surface. On the other hand, a natural source of amorphous silica could be used, for example, diatomaceous earth or diatomite. Diatomaceous earth can contain more than 90% silica, but also such minerals as clay, iron oxides, and other tiny mineral impurities are present. It is rarely used as a packing material for LC columns; more often it is used in gas chromatography (*e.g.,* Chromosorb stationary phase) [6]. Diatomite itself is a material originating from skeletons of diatoms. Diatoms [7] are microalgae that are found worldwide in both freshwater and marine environments. In addition, diatoms are common unicellular microscopic photosynthesizing algae that possess shells (exoskeletons) called frustules made of amorphous hydrated silica [8]. The siliceous walls of diatom frustules that are intricately patterned are decorated by an original pattern of ordered structural features such as pores, ridges, ribs, spikes, or spines, creating the most spectacular example of three-dimensional (3D) structured silica materials of biological origin. The pores of circular, polygonal, or elongated shapes range from a few hundred nanometers to a few micrometers in size. Based on symmetry in frustules morphology diatom frustules (biosilica) are composed of two overlapping valves called thecae (upper part – epitheca, lower part - hypotheca) with characteristic double-sided structure like a Petri dish [9-13]. Biosilica [14] is an inorganic polymer consisting of orthosilicate units formed by organisms such as diatoms or siliceous sponges. On one hand, in the classical approach, such particles

should be considered as irregular as they are not spherical, but on the other hand, they cannot be considered as irregular as all the particles have the same shape, which is even more important - the same size. Random observations using SEM revealed that the biosilica frustules population which we used is characterized by a narrower size distribution than commercially available Kromasil 100 of dp = 5 μm. We suspect that the complex influence of the frustules' parameters like valve diameter (ca. 4 μm), its sidewall thickness (ca. 1 μm), and thickness of the bottom (ca. 110-150 nm) in which 150-300 nm pores (holes) are present is responsible for quite good chromatographic properties. Particularly interesting may be the combination of the two last-mentioned features. Based on the considerations of Tallarek et al. [15] we can assume that those frustules positioned parallel to the column's long axis would show a rather small value of a characteristic length for hydraulic permeability while for those perpendicular ones – this value would equal frustule's diameter. There is, however, a significant difference when compared to spherical particles - the frustules are flat, and in the region of the pores (the holes) their thickness is around 110-150 nm, so perfusion through the frustules is possible. So, even if the frustule material would be totally porous (macroporous) , the characteristic length of the stagnant mobile phase would be very short, which would be comparable to superficially porous spherical particles or monolithic silica skeletons [16]. It is very likely that these features are responsible for high efficiency , high number of theoretical plates and relatively good permeability. This is due to good mass exchange between the analytes, the stationary phase and the mobile phase components.

$$R_s = \frac{\sqrt{N_2}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k'_2}{1+k'_2}\right)$$

where:

R_s : is the resolution between the two analytes.

N_2 : is the plate number of the second analytes.

α : is the separation factor between the two analytes.

k_2 : is the retention factor of the second analyte.

The diatom's frustule considers as a natural silica and the surface after removing organic deposition it contains a high level of silanol groups (Si-OH) and siloxane bond (Si-O-Si) *i.e.*, there is no metal impurities nor hetero atom and it would be promising from the point of view of "*green chemistry*" as the cultivation of diatoms is rather not environmentally harmful.

More than 100,000 living diatoms are classified by typical morphologies of their frustules that are unique to each of the diatom species [17]. Diatom frustules (biosilica) [18-20] serve as a source of inspiration for the creation of new nanostructured materials, and more recently, new nanomaterials have been created by maintaining the diatom nanostructure while changing the material chemistry. Also, frustules (biosilica) have been employed by numerous researchers to create different silica-material nano- and micro-composites because of their distinctive optical characteristics and large surface area. Due to the reactive silanol (Si-OH) groups that cover the surface, the valve surface of diatoms is easily modified by connecting with other substances [21]. Moreover, it has been noted that the diatom-derived bio-silica structure [22-23] possesses excellent mechanical and thermal durability, distinctive optical properties, and outstanding biocompatibility. The striking similarities between the morphology of frustules and that of artificially generated porous silica, like the low cost and the availability all around the world, make the nanostructured silica of diatoms an appealing source of material for several technological applications such as water treatment technologies, drug delivery system, transducer components, photovoltaic systems, bio-nanotechnology applications, and in solar energy harvesting systems [14].

These study shed light on the functionalization techniques that can be used to change the surfaces of biosilica and the possibility of using natural material as a filler/adsorbent-stationary phase liquid chromatography and extraction technique for the first time.

The Ph.D. thesis submitted for evaluation is based on four published or/and submitted for publication papers (D1- D4) from (JCR) list:

(D1) AL Saoud, H.; Sprynskyy, M.; Pashaei, R.; Kawalec, M.; Pomastowski, P.; *Buszewski B. Diatom biosilica: Source, physical-chemical characterization, modification, and application*. J. Sep. Sci. 2022, 45, 3362-3376.

(D2) AL Saoud H, Krakowska-Sieprawska A, Sprynskyy M, Pomastowski P, Buszewski B. *Nowe materiały na bazie 3D biokrzemionki.* Analityka. 2021;3:4–12.

(D3) M. Szumski, **H. Al Saoud**, I. Wojtczak, M. Sprynskyy, R. Gadzała-Kopciuch, S. Bocian, M. Dembek, M. Potrzebowski, B. Buszewski, *Diatom biosilica for the chromatographic purposes*. J. Chromatogr. A. (under review)

(D4) AL Saoud, H.; Szumski, M.; Sprynskyy, M.; Bocian, S.; Buszewski, B. *Biosilica as a new stationary phase in HILIC mode*. Chromatographia. (under review)

2. THE AIMS OF THE STUDY

The main aim of this study was to investigate the possibility of using and applying diatom frustules (biosilica) as a stationary phase for separation science, particularly for liquid chromatography. Another possibility has been to use obtained material as packing adsorbent for solid phase extraction mainly purification and preconcentration process technology. It would be promising from the point of view of "*green chemistry*" as the cultivation of diatoms is rather not environmentally harmful because it was extremely bure homogeneous natural material.

3. RESEARCH PROBLEMS

3.1 **Diatom biosilica as a promising material in the chromatography world**

Growing research interest in the use of biosilica results from its unique properties such as chemical inertness, biocompatibility, high mechanical and thermal stability, low thermal conductivity, and homogeneous porous structure with a large specific surface. Unlike the production of synthetic silica materials with a micro- or nanoscale structure in an expensive conventional manufacturing process, biosilica can be produced in huge quantities without significant expenditure of energy and materials. This fact makes it an unlimited, easily accessible, natural, inexpensive, and renewable material. Moreover, the production of biosilica is extremely environmentally friendly, as there is essentially no toxic waste, and the process does not require more energy compared to the production of synthetic silica-based materials. For all these reasons, biosilica are an intriguing alternative to synthetic materials in developing cheap biomaterials used in different branches of industry. For this a broad area, a comprehensive study summarizes the state-of art of biosilica materials, their characteristics approaches, and possible ways of application have been reported in the review articles entitled **" *Diatom biosilica: Source, physical-chemical characterization, modification, and application*" (D1)** and **"*Nowe materiały na bazie 3D biokrzemionki*" (D2).**

3.2 **Developing a new stationary phase for liquid chromatography**

The challenges of developing a new stationary phase have long been the subject of intensive research. Throughout the past several decades, numerous attempts have been made development within liquid chromatography has been to synthesize new stationary phases. Moreover, the evolution of new stationary phases has led to a new generation of selective adsorbents [24,25].Where many HPLC separations require packing materials with specific surface properties. For this reason, a variety of stationary phases has been developed; these include chiral Packings (e.g., cyclodextrin, bovine serum albumin (BSA), and Pirkle phases), chelating phases, and packings with short alkyl chains terminating in polar groups (e.g., $-NH_2$, $-NO_2$, -OH, etc.), liquid crystal molecules bonded to the silica surface, etc. [26]. In recent years, due to the unique properties of the frustule, diatoms are

an example of a new natural source of structural biosilica with appropriate properties to produce cost-effective biomaterials. Moreover, great possibilities are opening to change the physicochemical properties of the diatom silica by using modification methods for obtaining new silica functional materials with unique 3D structures preserved [27,28]. It is noteworthy here that under given growth conditions (growth medium, temperature, aeration, light conditions) the population of the obtained diatom cells is characterized by a very narrow size distribution and porosity. According to their physicochemical characteristics, diatom frustules are potential silica material for stationary phase chromatography and a filler for chromatographic columns. In this fact, the article entitled "***Diatom biosilica for the chromatographic purposes***" **(D3)** presents for the first time the successful use of diatom biosilica as a new stationary phase.

3.3 **Selective separation mode for a new stationary phase**

After the determination of the most efficient procedure to use biosilica for chromatographic purposes **(D3)**, the HILIC mode was applied in a further study entitled "***Biosilica as a new stationary phase in HILIC mode***"**(D4)**. The aim of this study was to use Hydrophilic interaction liquid chromatography (HILIC) as an alternative high-performance liquid chromatography (HPLC) mode for separating polar compounds[29]. Compared to traditional normal-phase liquid chromatography (NPLC) and reverse-phase liquid chromatography (RPLC), HILIC provides several distinct advantages. For instance, it is appropriate to analyze substances in complicated systems that consistently elute close to the void in reserved-phase chromatography. Polar samples exhibit strong solubility in the aqueous mobile phase employed in HILIC, which outweighs the limitations of the frequently encountered poor solubility in NPLC. For HILIC separations, any polar chromatographic surface may be utilized. Classical bare silica or silica gels that have been heavily modified with polar functional groups make up the majority of HILIC stationary phases [30] as we mentioned in successfully using diatom biosilica in chromatography **(D3)**. There are excellent opportunities to alter the physicochemical characteristics of diatom biosilica by applying modification techniques to create novel silica functional materials with retained distinctive 3D structures. According to their physicochemical

characteristics, diatom frustules are a potential silica material for the chromatographic stationary phase and a packing for liquid chromatographic columns.

3.4 **Biosilica as packing material in solid phase extraction**

Extraction techniques are widely used, particularly those based on phase-based processes such as liquid-liquid and/or liquid-solid. They have become the most widely used in routine determinations due to the reproducibility of data, precision, relatively low cost of appropriate analysis, simplicity of the determination, and the possibility of a direct combination of those techniques with others [31,32] . For this purpose, a new carrier material was used for these purposes for the first time in extraction and it was a simple, rabid, environmentally friendly, and highly efficient extraction technique with water as an extraction solvent for the isolation and purification of analytes contained in complex mixture, *i.e.* polycyclic aromatic hydrocarbons (fluoranthene, benzo(k)fluoranthene, benzo(b)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, indeno(1,2,3-cd)pyrene and/or biologically active substances *e.g.* mycotoxins, polyphenols, flavonoids, *etc*. In order to change selectivity, in capillary or in chips [33]. The idea is to use various sorbents based on biosilica located in a container having a form of a piece of fused silica capillary (miniaturization) to connect it *on-line* to LC column to create a nanoSPE-nanoHPLC system. The construction proposed here was built on the basis of the previous results described by Grzywiński et al. Briefly, it consists of two 10-port nanoLC valves designed for connection of 360 um fused silica capillaries. The scheme of the connections is presented in Fig. 1. In this special arrangement the eluate from the extraction column is passed through the detector during the extraction step, so any possible loss of the analytes that may occur due to the extraction column breakthrough can be observed. After the extraction step is complete, the change of the valve position makes the mobile phase flush the extraction column, so the analytes are desorbed and on-line transferred to the separation column. Simultaneously, the valve changes the stream of the liquid driven to the detector to the separation column mobile phase. So, the final chromatogram can be divided into two sections – the extraction part and the separation part, which turned out to be very convenient. The only drawback of the system may be increasing the extra column volumes

which might result in non-symmetric peaks and, sometimes, deteriorated resolution.

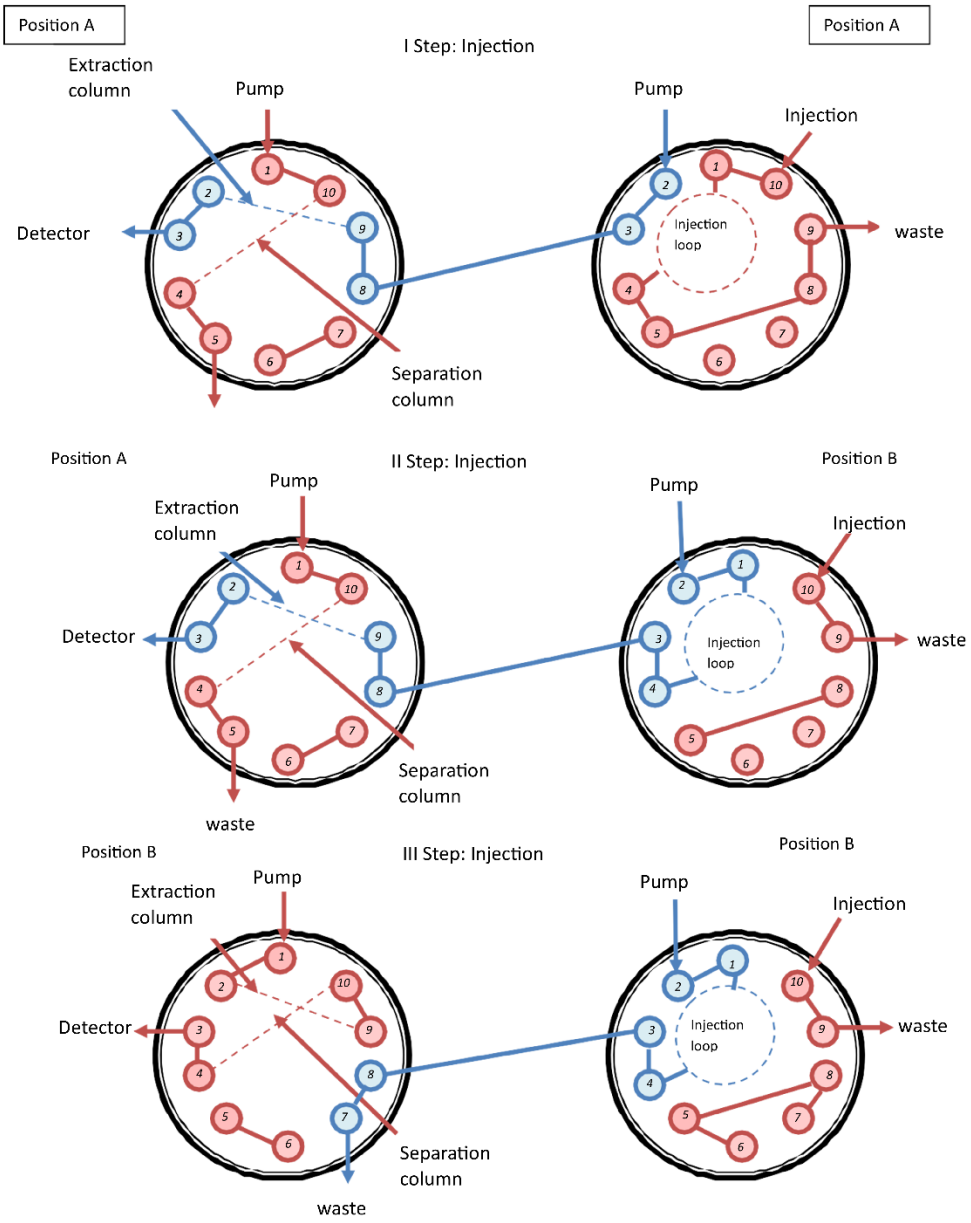

Figure. 1 Scheme of the valve system in µSPE–nanoHPLC

The model solution of a set of sex poly cyclic hydrocarbon in water was injected and the extraction and separation procedure were started. The result of such a chromatographic process is presented in Fig. 2. In this case we didn't observe any breakthrough of the extraction bed , and the analytes were transferred to the C18 separation column.

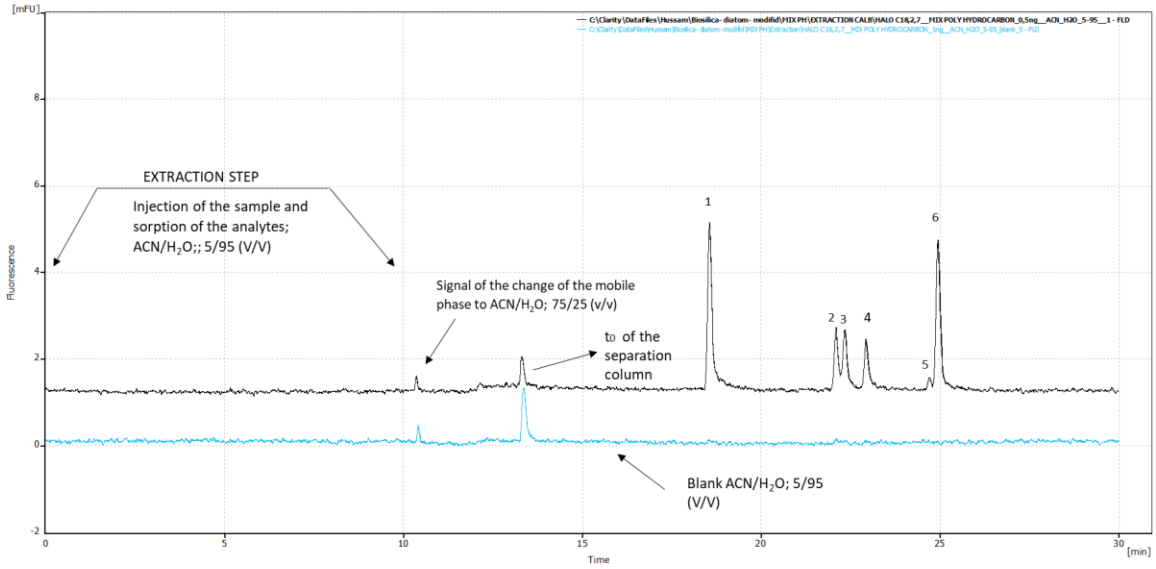

Figure 2. Example chromatograms of nanoSPE-nanoHPLC process of blank solution and solution containing six polycyclic aromatic hydrocarbons. Chromatographic conditions: sample solvent 5/95 ACN/H_2O, flow rate 5µl/min, extraction time 10 min, elution solvent/mobile phase 75/25 ACN/H_2O, flow rate 5µl/min, gradient 0-10 min 75/25 ACN/H_2O,10-22 min increase from 75-100 CAN, 22-30 min 100%ACN. elution order: 1 - fluoranthene, 2 - benzo(k)fluoranthene, 3 - benzo(b)fluoranthene, 4 - benzo(a)pyrene, 5 - benzo(ghi)perylene, 6 - indeno(1,2,3-cd)pyrene).

3.5 **Surface modification and preparation of biosilica**

The 3D-structured diatom biosilica was obtained under laboratory conditions by the cultivation of the selected species of *Pseudostaurosira trainorii*. This diatom species was provided by the Culture Collection of Baltic Algae, Institute of Oceanography, University of Gdansk, Poland. Microalgae were cultivated using Erlenmeyer flasks (5 L) with a Guillard's growth medium that contained silicon concentration of 7 mg/L under air aeration and light regime 12h light/12h darkness. After growing diatom cells were separated by filtration using a vacuum pump and washed with distilled water. Diatom biosilica (diatom exoskeletons) was isolated from dried diatom biomass by decomposition of organic matter with hydrogen peroxide and hydrochloric acid. The frustules obtained in this way were washed with distilled water and dried in the oven at 120°C. Chemical modification of diatom frustules were performed according to the methodology used for silica gel modification described by Buszewski et al.[34] Before chemical modification of the diatom frustules, a sample of the adsorbent was placed in a glass reactor specially designed to protect the reagents from contact with the external environment. Diatom

frustules were dried at 180°C under vacuum for 24 h to remove physically adsorbed water. The temperature was then reduced to 80°C, and octadecyldimethylchlorosilane dissolved in anhydrous toluene was added. In order to bind hydrogen chloride generated during the reaction, triethylamine was added to the reactor. After 12 h, the reaction product was washed with toluene, ethanol, hexane and acetone. Then the biosilica was dried in a vacuum oven for 24 h at a temperature of 70°C. According to our results in this study "***Diatom biosilica for the chromatographic purposes***"**(D3)** and "***Biosilica as a new stationary phase in HILIC mode***" **(D4)** it turned out it was an efficient method to prepare and modify the biosilica.

3.6 **Column packing procedure**

At first the fused silica capillary section of 3 m in length was consecutively washed with 2 mL of each of the following solvents: dichloromethane, acetone, water, 1M sodium hydroxide, water, and acetone. Then the capillary was cut into 20 cm sections and in each section the outlet ceramic frit was synthesized using sodium water glass (750 μL) solution in formamide (120 μL) which was introduced by capillary forces to the 2.5 cm distance from the outlet. Then both ends of the capillary were plugged with pieces of silicone rubber and the whole was heated in the oven at 100°C for 1 hour. After that, prepared porous frit was flushed with water and acetone and dried in the stream of nitrogen. 50 mg of biosilica was weighed out and suspended in 470 μL of isopropanol. The mixture was then homogenized and degassed by sonication for 10 min. The slurry was transferred to a stainless-steel reservoir (100 x 2 mm) with the empty capillary column connected to its outlet. Such a prepared set-up was connected to a high-pressure air-driven pump (model DSF-122, Haskel, Burbank, USA). The slurry was pushed with methanol at a pressure of 20 MPa for 2 hours. After that, the column was left to depressurize slowly. Finally, it was gently disconnected from the reservoir and the outlet ceramic frit was cut to its final length of 0.5 cm. According to our results in this study "***Diatom biosilica for the chromatographic purposes***" **(D3)** and "***Biosilica as a new stationary phase in HILIC mode***" **(D4)** it turned out it was a rapid, efficient method to pack the column.

4. PUBLICATIONS

The detailed description of the methods used, obtained results, their discussion, and conclusions are presented in two unpublished experimental papers which are currently under peer review in the Journal of Chromatography A (D3) (submitted on May 6, 2023) and Chromatographia (D4) (submitted on March 24, 2023).

(D1) AL Saoud, H.; Sprynskyy, M.; Pashaei, R.; Kawalec, M.; Pomastowski, P.; Buszewski B. Diatom biosilica: Source, physical-chemical characterization, modification, and application. *J. Sep. Sci*. 2022, 45, 3362-3376.

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REVIEW ARTICLE

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Diatom biosilica: Source, physical-chemical characterization, modification, and application

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Growing research interest in the use of diatomaceous biosilica results from its unique properties such as chemical inertness, biocompatibility, high mechanical and thermal stability, low thermal conductivity, and homogeneous porous structure with a large specific surface. Unlike the production of synthetic silica materials with a micro- or nanoscale structure in an expensive conventional manufacturing process, diatomaceous biosilica can be produced in huge quantities without significant expenditure of energy and materials. This fact makes it an unlimited, easily accessible, natural, inexpensive, and renewable material. Moreover, the production of biosilica is extremely environmental friendly, as there is essentially no toxic waste and the process does not require more energy compared to the production of synthetic silica-based materials. For all these reasons, diatoms are an intriguing alternative to synthetic materials in developing cheap biomaterials used in a different branches of industry. In this review, the state-of-art of biosilica materials, their characteristics approaches, and possible ways of application have been reported.

KEYWORDS
application, biosilica, characterization, diatom, silica

1 | INTRODUCTION

Silica is one of the most well-known inorganic polymeric materials [1]. There are different groups of silica materials including ordered mesoporous silicas, amorphous silicas, fumed silicas, and silica gels [2]. Porous silica is usually used to support the preparation of silica-based column packings with chemically bonded phases [3]. Silica is found in various forms, all of which have the same stoichiometric composition of SiO_2 and its hydrated form $SiO_2 \cdot nH_2O$ [4]. In addition, the silica (SiO_2) particle is thought to be made up of an uneven three-dimensional network of SiO_4 tetrahedra [5, 4]. The angle between the (-O-Si-O-) bond is 109.5°, and the ≡Si-O-Si≡ bond is typically 147°, although the siloxane bond can range from 120 to

Abbreviations: AFM, atomic force microscopy; DB, diatom biosilica; DE, diatomaceous Earth; FTIR, Fourier-transform infrared spectroscopy; ICP, inductively coupled plasma; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; TEM, transmission electron microscopy; TGA, thermogravimetric analysis; UV-VIS, ultraviolet–visible spectroscopy; XRD, X-ray powder diffraction.

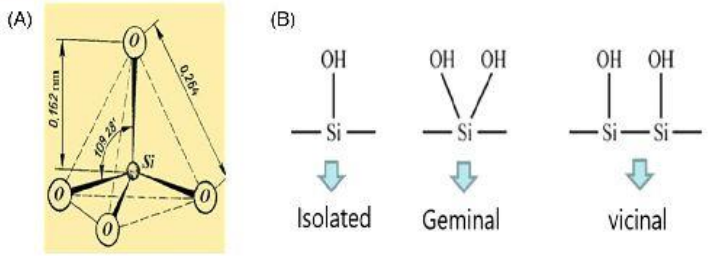

FIGURE 1 Structure of silica (A) and types of active centers (B)

180° depending on the energy of the bonds [6]. Because the ≡Si-O- bond length is about 0.162 ± 0.002 nm, which is much lower than the sum of the covalent radii of silicon and oxygen atoms (0.191 nm), the short bond length largely accounts for the partial ionic character of the single bond and is responsible for the relatively high stability of the siloxane bond [4, 7]. The silica lattice is usually three-dimensional, depending on how the tetrahedra are linked. (Figure 1A). There are various active sites on the surface of silica (Figure 1B), which are silanol groups. In general, three types of silanol groups can be distinguished: isolated (total content: 6–19% of all surface silanols), vicinal (bridge) (60–65%), and geminal (10–12%), where vicinal silanols are formed by hydrogen bonds between neighboring silicon atoms, whereas geminal silanols are bonded to the same silicon atom [4, 8]. Furthermore, siloxane groups are found on the surface of silica, accounting for 20–35% of the total amount of silanol groups generated because of the dehydroxylation of the hydroxyl group [9]. Silica has appealing features, such as adsorption capacity, acid/base chemistry, and thermal stability, which can be exploited. Silica can be grafted with a range of functional groups, resulting in a significant enrichment of their surface properties [4, 5, 8, 10]. Silica was widely utilized in various industries [4] including heterogeneous catalysis [4, 11], microelectronic devices [12], and optical fibers [13]. In addition, because of its high mechanical strength, good chemical stability, high-temperature resistance, easy dispersion in solvents, and other unique characteristics, silica was widely used as functional fillers, catalyst supports, high-performance ceramics, and chromatographic column fillers [4, 5, 10, 14]. Diatom biosilica (DB) is another type of silica. DB is reactive silica extracted by a particular process from diatom biomasses and represents the highest specific strength of any known biological material [15]. Also, DB consists primarily of amorphous, hydrated SiO_2 (silica) containing a small proportion of organic macromolecules that have long been speculated to control the deposition of silica and nanopatterns [16, 17]. DB shells last long after the diatoms die and after their internal organic matter has disintegrated and decayed. As a result, many places that were diatom rich in ancient periods (e.g., oceans, lakes, and marshes) contain sedimentary rocks primarily formed of DB because of geographical and environmental changes [18]. In addition, biosilica is an inorganic polymer formed by organisms, such as diatoms or siliceous sponges of orthosilicate units, in which two silanol groups are joined together to one bond or siloxane [19]. Diatoms are a broad group of single-celled microalgae noted for their silica-based cell walls that exhibit precisely ornamented morphologies with patterns of nano- to micrometer-sized pores (~15,000 identified and >100,000 approximate species) [15]. Diatoms amorphous silica cell walls are known as frustules that are identically repeated from generation to generation [20]. Marine diatoms and sponges in nature can direct the synthesis within their cell walls of complex structures through complex molecular methods that regulate the transport and deposition of silica [21]. Several diatom species with different frustule shapes and architecture are shown in Figure 2 [22]. DB consists of two different building blocks: valves produced during cell division and girdle bands produced during interphase processes [18]. In the last decade, research interest in the use of DB for biomedical applications has grown [23]. Recognition that algae may be a source of different forms of metabolites or possible drug molecules that are not present in higher plants or other conventional drug supplies is the primary reason for this increase in interest [24]. Furthermore, DB also provides an opportunity to search bio-inspired photonic crystal structures in photovoltaic systems for light trapping [25], it is also possible to use these low-cost and largely usable natural materials as transducer components for optical biosensors or as targeting microcapsules for drug delivery. DB formation is a well-established model system for elucidating biomineral morphogenesis molecular pathways and is of growing importance as a material for bionanotechnology applications [26]. Besides, the overall results indicate that DB

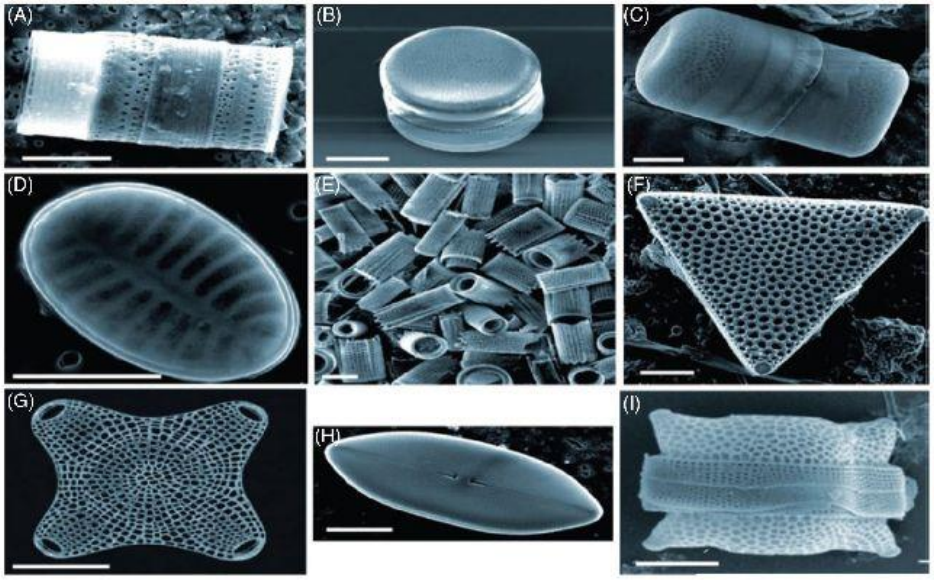

FIGURE 2 The extraordinary diversity of shapes and structures in diatoms. (a–d) and (f–I) SEM images of several marine diatom species. (e) SEM of fossilized diatom biosilica structures from diatomaceous earth (Diatomite mine NSW, Australia). Scale bar: 10 μm

provides a specific forum for in-depth research into the role that nanotopography and chemistry play in biomedical applications [20, 23]. Also, natural DB from diatoms is a suitable biocompatible material for medicinal applications [27]. Diatoms, because of their optical properties, are sometimes referred to as jewels of the sea or living opals [28]. Biosilica is used for numerous applications, such as water treatment technologies [29] and as a drug delivery vehicle [30]. Furthermore, biosilica can shield DNA from UV radiation disruption, and this phenomenon will flourish in future applications in solar energy harvesting systems [29].

2 | SOURCE OF DIATOM BIOSILICA

There are two sources of DB: diatomaceous earth (DE) or diatomite and living diatom cells [19]. Diatoms can be easily produced by cultivation as a source of natural DB [31]. In addition, diatoms microalgae can be considered living factories, producing nanostructured and mesoporous DB shells (frustules) with a highly ordered hierarchical architecture [32]. What is more, frustules obtained from marine habitats are also an excellent source of biosilica that is cost-effective [23]. Diatoms are a natural source of biosilica and can be grown in photobioreactors under environmental friendly conditions on a large scale. In addition, different types of diatom species were used to obtain biosilica (Figure 3A and B), for example, *Pseudostaurosira trainorii* [29, 33], live diatoms *Navicula species* [34], *Pinnularia species*, *Coscinodiscus wailesii* [35], *Thalassiosira weissflogii* [25], and *Aulacoseira species* [36]. *Pinnularia diatom cells* were grown to the desired cell density in a two-stage photobioreactor growth method, and then titanium was metabolically inserted into the frustule biosilica [37].

DE or diatomite consists predominantly of particles of DB (>90%), which are largely fragmented and structurally distinct since they come from several different species of diatom [38]. Generally, the structure of formed diatom frustules is classified into the following models according to the shape (Figure 4) [39]. In addition, diatomite is a sedimentary source, consisting of the accumulation of the skeletons produced by the diatoms as a protective covering. The skeletons are essentially hydrated or opaline silica amorphous; they can also contain alumina [40]. Also, diatomaceous biosilica is one of the most used replacements for mesoporous silica products in modern technologies because of its three-dimensional, porous form, wide availability, and the potential of biosynthesis through the cultivation of diatoms under artificial conditions [41].

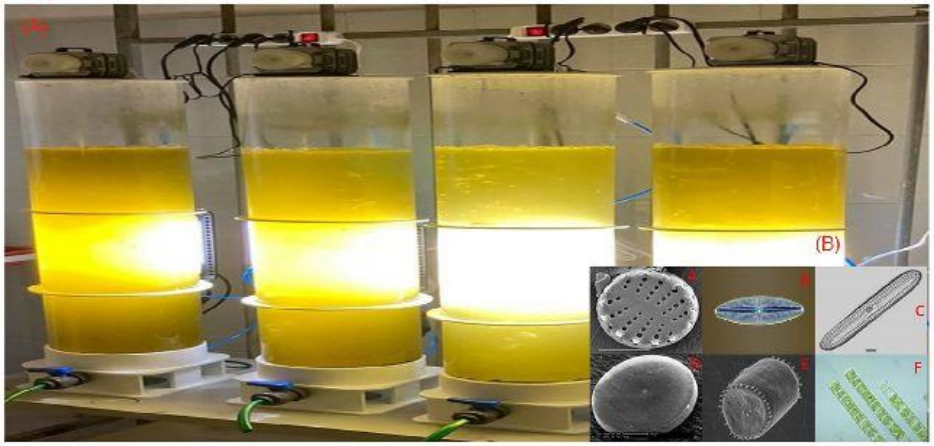

FIGURE 3 Cultivation of diatoms in photobioreactors under laboratory conditions (A), different types of diatom species were used to obtain biosilica (B); (a) *Pseudostaurosira trainorii*, (b) *Navicula* sp., (c) *Pinnularia* sp., (d) *Coscinodiscus wailesii*, (e) *Thalassiosira weissflogii*, (f) *Aulacoseira* sp

FIGURE 4 Structure of the diatom frustule, 3D models [39] (with permission from Springer)

3 | CHARACTERIZATION OF BIOSILICA

3.1 | Scanning electron microscopy

SEM can give surface topography, crystalline structure, chemical composition, and electrical activity of the top 1 μm layer of the material. Many specific microscope stages (e.g., hot, cold, or built to permit in situ mechanical testing) can be used to enable examining the behavior of the sample under different conditions [42]. Sprynskyy et al. used SEM-EDX to provide a description of the structure, morphology, and composition of diatom frustules that have been cleaned, where they selected DB was generated by the cultivation of the selected species of diatoms *P. trainorii*, and they found the pore size in the range of 150–200 nm, the average diameter of valves in the frustules is about 4–5 μm, the gaps between arranged rows are near 450 nm, and the space between the individual pores in the rows is about 130 nm [43]. Figure 5 shows a variety of silica structures produced by living diatoms [44]. Also, Yu et al. pointed out that SEM is necessary for the visual identification of diatoms, as the size of a diatom ranges from a few micrometers to 0.5 mm [45]. One of the main obstacles in classifying diatoms under SEM is the absence of literature endorsing them [46].

3.2 | Transmission electron microscopy

Transmission electron microscopy (TEM) was used to understand better the function of the frustule including its filtering capacity (Figure 6) [38]. Furthermore, TEM was utilized to investigate the micro- and nanoscale structures of diatom frustules *P. trainorii* and the porous structure and elemental composition of the frustules [43]. In addition, TEM was used to examine the surface morphology, microstructure, and crystal structures of the *diatom Psammodictyon panduriforme* [47]. Moreover, TEM was utilized to investigate the influence of varied Au-NP loadings on to biosilica of *Thalassiosira pseudonana* [48]. Also, TEM [49] was used to study the *Claviceps fusiformis* by using the high-pressure freezing method and discovered that the nonsilicified is made up of two thick layers, each of which is made up of a fibrillar meshwork.

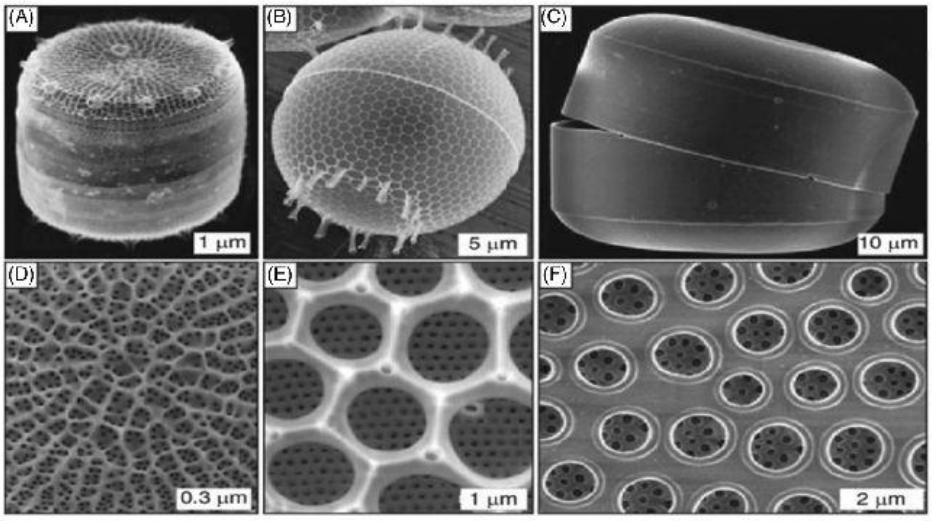

FIGURE 5 Scanning electron microscopy images of silica produced by living diatoms. (a, d) *Thalassiosira pseudonana*, (b, e) *Stephanopyxis turris*, and (c, f) *Coscinodiscus granii* [48]

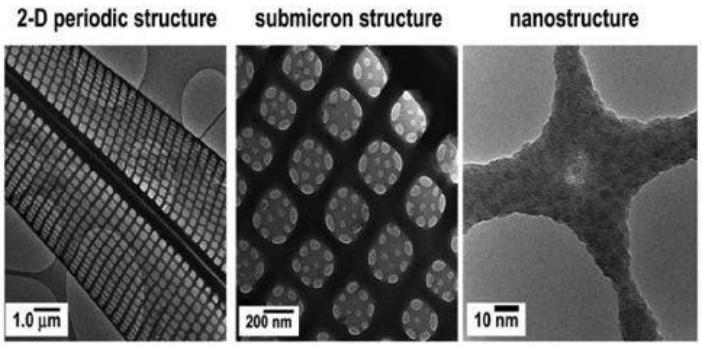

FIGURE 6 Transmission electron microscopy (TEM) images of biosilica frustules isolated from the diatom *Pinnularia* sp. Left image: Submicron pore structure; middle image: nanoscale fine pore structure; right image: biosilica nanostructure within the fine pores [38] (with permission from Royal Society of Chemistry)

3.3 | Atomic force microscopy

Atomic force microscopy (AFM) was used to study the DB obtained from cultivated diatoms of the species *P. trainori*, and they found that the shells of diatomaceous organisms are elliptical, flattened spheroids, and the size of the frustules varies from 2 to 10 μm [50]. Moreover, AFM was utilized to investigate the frustule topography of two diatoms species, *Coscinodiscus species* and *Thalassiosira eccentrica*, where they obtained detailed information about porous biosilica nanostructures (Figure 7) [51]. In addition, AFM was used to get imaging of diatom cell wall nanostructures and showed, for example, the features of the native mucilage on the external surface of the exoskeletons, the biosilica and silica spheres nanostructure, the ordering of mesopores under differing culture conditions, and the mechanical properties [52]. Besides, AFM is an excellent method of microscopy for imaging nano-sized

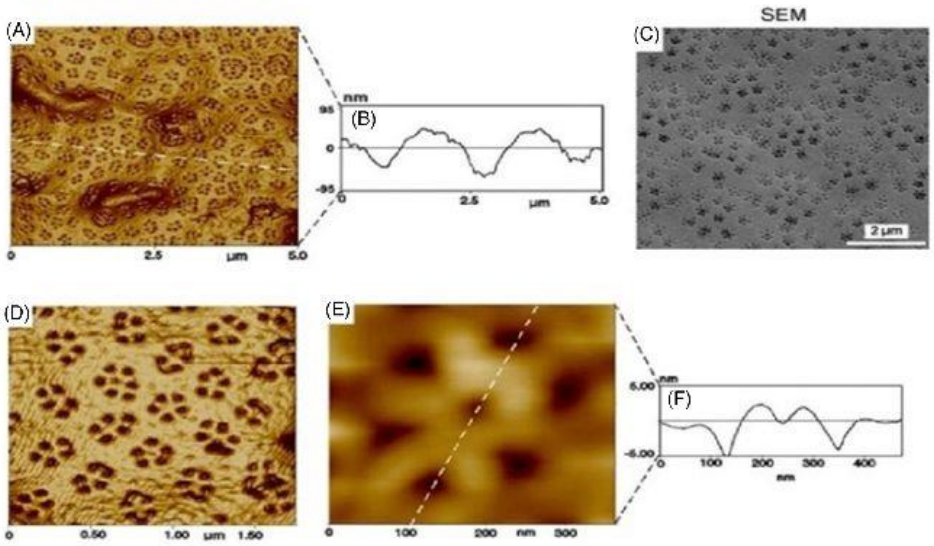

FIGURE 7 Outer surface (cribellum) of *Coscinodiscus* sp. (A) AFM image (tapping mode in air) of the cribellum surface showing porous domes forming hillock topography. Corresponding cross-section graph in (B). (C) Comparative SEM image of cribellum surface. (D) Enlarged AFM image of a single dome shows details of pore organisation, z-range 100 nm. (E) High-resolution AFM image of one typical hexagonal pore array and (F) corresponding cross-section graph [51] (with permission from Springer)

objects, such as diatoms, allowing for high image resolution, based on small variations in surface height and on the imaging of translucent materials [53]. In addition, Pletikapić [49] proposed that a primary field in which AFM could be implemented is high-resolution mapping of organic and inorganic domains in diatoms.

3.4 | Nuclear magnetic resonance

NMR was used to study the biosilica from *T. pseudonana* to improve the understanding of diatom cell walls of this substance's structure and composition, as shown in Figure 8A and B, where Figure 8A refers to the spectrums of the separate signals for Q2, Q3, and Q4 groups of the condensed biosilica and, Figure 8B refers to the superimposition of the spectral contributions of all organic components associated with the biosilica [54]. These signals have ^{29}Si chemical shifts centered at $\delta = -92$ ppm, $\delta = -100$ ppm, and $\delta = -108$ ppm [55], which correspond to the Q2, Q3, and Q4 groups, respectively. Which, in turn, correspond to geminal silanols, free silanols, and siloxane groups, respectively [56]. The correlation signals in the ^{13}C dimension are in the region of C-N bonds at roughly $\delta = 45–47$ ppm and $\delta = 55–57$ ppm [54] and in the region of alkyl carbon at $\delta = 20–70$ ppm, region of C-OH at $\delta = 70–80$ ppm, region of aromatic and C = C at $\delta = 105–140$ ppm, and region of C = O at $\delta = 170–180$ ppm. Also, NMR was utilized to understand the physicochemical properties of biosilica and bioinspired silica [57]. In addition, NMR was used to obtain the metabolic profiles of the polar *diatom fragilariopsis cylindrus*, leading to the discovery of a novel metabolite in this organism [58]. Furthermore, NMR [59] was utilized to describe and prove the viability of DB for drug delivery.

3.5 | X-ray powder diffraction

X-ray powder diffraction (XRD) was used to describe diatomite composition and mineral contamination [60]. Also, XRD was utilized to proof the contribution of calcium carbonate to the formation of deviline, originally present in the diatomite [61]. In addition, the XRD method was used to analyze the diatomite support crystalline phases and the composites of TiO_2/diatomite calcined at various temperatures [62]. Besides, XRD was utilized to characterize the thermally prepared biosilica by performing the analysis of the crystallization phase or the amorphous state [63]. XRD was applied to describe the degree of drug

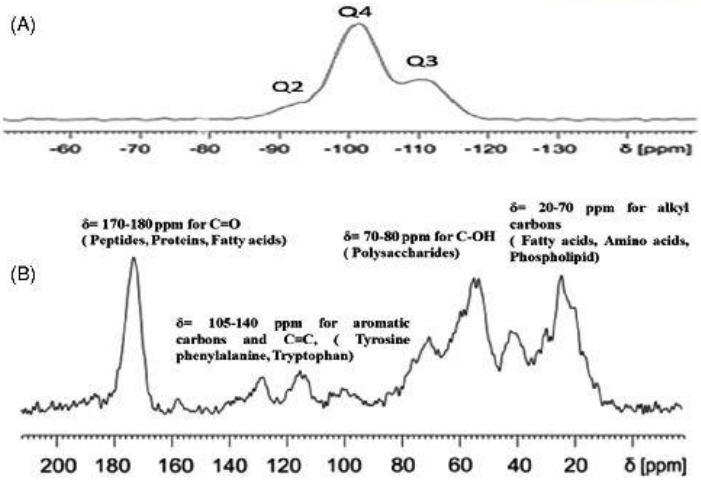

FIGURE 8 29Si CP MAS NMR spectrum (A) and 13C CP MAS NMR spectrum of biosilica from *T. pseudonana* (B) [54] (with permission from Elsevier)

crystallinity of biosilica and Ca-biosilica specimens [64]. Also, the XRD technique was used to study [65] the DB, which is a cultivation from diatom species *P. trainorii*, and they found that biosilica was identified as hydrated silica like opal-A. Opal-A is a noncrystalline form of hydrous silica ($SiO_2 \cdot nH_2O$) that often consists of submicrometer-sized spheres. In addition, the spheres in opal-A consist of 25–40 nm-sized subparticles and are most likely to formed by an aggregative growth process [66].

3.6 | Ultraviolet-visible spectroscopy

UV-VIS spectroscopy analysis was used to study the optical properties of diatom silica frustules, and they verified that when irradiated with UV radiation, the diatom *Cyclotella meneghiniana* exhibits luminescence in the blue portion of the electromagnetic spectrum and this species of diatom absorbs UV light with peak absorption at 274 nm [67]. In addition, the UV-Vis spectroscopy technique was utilized to determine that biosilica reinforced polybenzoxazine composites had good UV shielding behavior [68].

3.7 | Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) was used to give beneficial information about the DB. In addition, FTIR analysis was used to indicate that the diatom surface reveals sharp peaks about 1049–1060 cm^{-1} that are characteristics of the ≡Si-O bond, and the association of inorganic and organic materials with the diatom surface contributes to the change in the absorption band of ≡Si-O, ≡Si-OH, and ≡Si-O-Si≡ [69]. Furthermore, FTIR is an analytical technique that has also been extended to diatoms, allowing for bulk chemical composition experiments without or with a limited chemical alteration [70]. Moreover, FTIR-attenuated total reflection analyses were used to prove the presence of the functional groups' characteristic for amorphous silica framework such as opal-A as well as amine and amide groups of the organic residuals related to biosilica-associated proteins. Therefore, the obtained biosilica can be considered as a naturally organic functionalized 3D material [43]. Also, FTIR was used to study dry SND (silver nanoparticles templated on diatom frustules) and raw diatom frustules and they found that the stretching, and bending vibrations of the -OH groups, which are mainly exterior silanol groups (Si-OH) of the amorphous silica, are seen at 3404 cm^{-1} for SND and 3445 cm^{-1} for raw diatom sample, asymmetric stretching modes of CH_2 cause bands at 2918 and 2926 cm^{-1} for SND and raw diatom, respectively. The symmetric stretching mode of CH_2 is responsible for the 2853 and 2839 cm^{-1} bands for SND and raw diatom, respectively. Stretching and bending vibration of -OH groups cause peaks at 1649 and 1634 cm^{-1} for SND and raw diatom, respectively. Peaks at 1376 and 1383 cm^{-1} for SND and raw diatom, respectively, are due to C = O stretching vibration carboxyl groups present in diatom cells. Peaks at 1029 and 1036 cm^{-1} for SND and raw

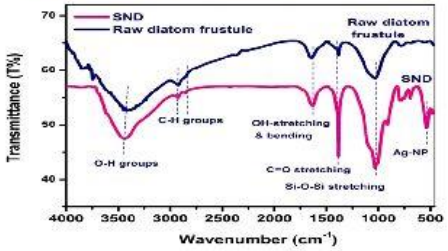

FIGURE 9 FTIR spectra of SND (Ag-NPs templated on diatom frustules) and Raw diatom frustules showing different functional groups [72] (with permission from Elsevier)

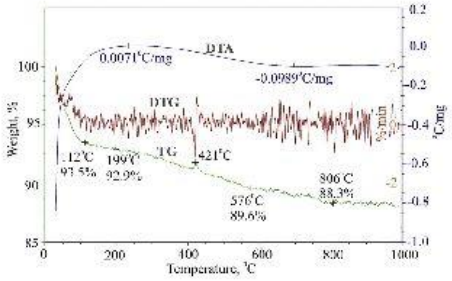

FIGURE 10 Phase transformations and thermal stability of the diatom biosilica [43] (with permission from Elsevier)

diatom are owing to asymmetric (≡Si-O-Si≡) vibration in the silica-based diatom frustule (Figure 9) [71].

3.8 | Inductively coupled plasma

Inductively coupled plasma (ICP) was used because it is a cost-effective and efficient approach for biomass processing optimization for the development of three species of marine diatoms that have been carried out [72]. In addition, ICP MS was used to determine the elemental composition of the frustules [35]. Furthermore, ICP optical emission spectrometry was utilized to test the diatom frustules' bulk composition for heavy metal contamination [22]. Moreover, ICP analysis used to determine the bulk concentration of germanium in frustule biosilica [73]. Also, ICP was used to study the centric diatom *T. pseudonana* to determine molar element to silicon ratios for several foreign elements that are found in the biosilica [74].

3.9 | Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization TOF MS (MALDI-TOF MS) is a high-throughput technique focused on comparing the molecular fingerprints acquired by microbial cells with the reference spectra database using different algorithms implemented into accessible devices [75]. MALDI-TOF MS is ideal for use in microbiology laboratories, where it functions as a paradigm shifting, rapid, and robust tool for precise microbial identification [76]. MALDI-TOF MS was used to research the overall structural characteristics of native extracellular polysaccharides formed by diatoms [77]. In addition, the MALDI-TOF MS technique [78] was used to classify diatoms by studying silica-associated lipids, chlorophylls, silaffins, and polyamines.

3.10 | Thermogravimetric analysis

Thermogravimetric analysis (TGA) was used to give data on the grafting to the surface of the diatom of 3-mercaptopropyl-trimethoxysilane, 3-aminopropyl-trimethoxysilane, and *n*-(2-aminoethyl)−3-aminopropyl-trimethoxysilane [79]. Besides, TGA was used to describe the removal of physisorbed water and photoluminescent surface silanols on the biosilica as a function of temperature [80]. In addition, the TGA was utilized to study the thermal stability and phase transformation of the DB of the selected species of diatoms *P. trainorii*, and they found three different phases of mass loss that may stand out in the heating process, as shown in Figure 10 [43].

3.11 | Low temperature nitrogen gas adsorption/desorption

The use of nitrogen adsorption for pore size analysis dates to the late 1940s and is based on the Kelvin equation with a correction for the multilayer thickness on the pore walls [81]. The IUPAC classified the adsorption or desorption isotherm into combination types I and II [82]. Where type I refers to the property of microporous materials with small exterior surfaces and type II refers to nonporous or macroporous materials [65]. In addition, N2 adsorption or desorption isotherms show that the virtually nonporous structure of diatom frustules becomes mesoporous when the organic matrix was removed [83]. By using the low-temperature nitrogen gas adsorption or desorption possible to calculate the following parameters of the porous structure of diatomites, such

TABLE 1 Parameters of the porous structure of diatom

Diatom species	Pore size	BET (m^2/g)	Refs.
Bare silica gel	20	361	[3, 5, 84]
Pseudostaurosira trainorii	150–200	16.9	[43]
Amphora sp.	–	11	[85]
Biosilica	30 nm	3.93	[65]

as surface area (S), pore volume (V), pore diameter (D), and pore size distribution, using theoretical adsorption models include Brunauer–Emmett–Teller, Barret–Joyner–Halenda, density functional theory, and Korváth–Kawazoe (HK) models [43], as shown in Table 1.

4 | MODIFICATION AND FUNCTIONALIZATION OF DIATOM BIOSILICA

Diatom frustules (biosilica) are useful in various fields due to their ability to be easily modified with different molecules [23]. Furthermore, using substitution reactions between the organosilanes and silanol moieties present on the silica surface, silica and other supports can be modified with different functional groups, resulting in stable covalent bonds [8]. Cicco et al. used classical silanization techniques to chemically modify the biosilica surfaces, yielding diatom silica microparticles functionalized with 3-mercaptopropyl-trimethoxysilane and 3-aminopropyl-triethoxysilane [86]. While Lang et al. replaced the soluble silica substrate for frustule formation, $Si(OH)_4$, with a 3:1 molar ratio of tetramethoxysilane and 3-mercaptopropyltrimethoxysilane in the in-vivo functionalization [87]. In addition, the biosilica shells were functionalized with the 2,6,6-tetramethylpiperidine-*N*-oxyl molecule, a reactive oxygen species scavenger, resulting in the formation of an antioxidant nanoporous scaffold for osteoblast-like cell development, drug delivery, and implantology [88]. Losic et al. used dopamine-modified magnetic nanoparticles (iron oxide, Fe_3O_4) to functionalize the surface of diatom structures and introduce diatoms with magnetic properties [89]. Furthermore, the diatoms were functionalized with graphene oxide by Kumeria et al., which improved the photoluminescent properties of the diatoms [27]. Moreover, Selvaraj et al. used some amine-passivated frustules of the diatom *Nitzschia sp.* to detect nitroaromatic compounds with high sensitivity and specificity [90]. Furthermore, chemical functionalization of the biosilica surface can be easily performed using silane coupling reagents to enable a variety of surface functions (hydroxyl/amine/thiol/carboxyl groups) as well as targeted modification of ligands by functionalization with moieties containing hydrophobic, hydrophilic, that is, C18, amino, cyano, phenyl, cholesterol, etc (Figure 11) [4, 8]. In addition, Leonardo et al. summarize the ways for assembling diatoms onto solid substrates, modifying their properties using physical, chemical, or cultural approaches to produce composite frustules or replicas, and/or functionalizing them with biomolecules presented in Table 2 [91].

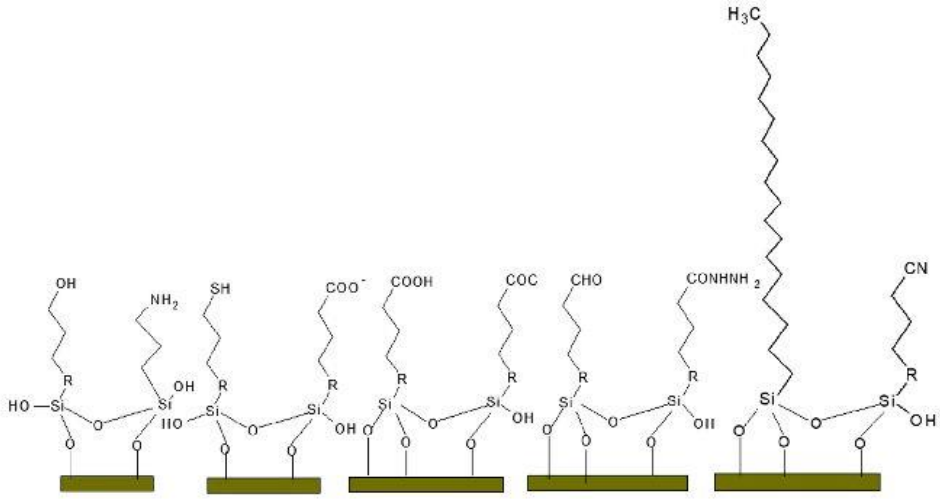

FIGURE 11 Modified biosilica with different functions

TABLE 2 Modification and possible applications of diatom frustules

Diatom	Coated material	Modification	Possible applications	Refs.
Aulacoseira	Fe_3O_4	Fe_3O_4 via sol-gel process	1. As removal from water 2. Drug carrier	[92] [89]
Aulacoseira sp.	TiO_2	TiO_2 via gas/solid displacement reactions	Solar cell	[25, 93]
Thalassiosira. eccentric	Au	Au via thermal evaporation	1. Localised surface 2. Catalyst	[94] [95]
Thalassiosira. eccentric	TiO_2	TiO_2 via ALD	1. H_2 sensor 2. Photocatalysts	[96] [97]
Pinnularia sp.	Ag	Ag via self-assembly	1. SERS detection of rhodamine 6G 2. Electroluminescence	[98] [99]
Coscinodiscus	Au	Au via surface functionalization combined with electroless deposition, evaporation	Extraordinary optical transmission (EOT)	[100]
Coscinodiscus	MnO_2	MnO_2 via template-assisted hydrothermal process	Supercapacitors	[101, 102]
Synedra	Ag	Ag via thermal evaporation	SERS substrates	[103]

5 | APPLICATION OF BIOSILICA

DB is an important point for the applications of the modern era such as for drug delivery applications [104], gene therapy as nucleic-acid delivery systems [23]. In addition, attempts have been made to recognize them as being of decisive value in commercial and industrial applications such as carbon-neutral fuel synthesis, health foods, biomolecules, and nanotechnology-related materials [105]. Furthermore, DB has recently been used and considered for a wide variety of applications by highly technical industries [106] such as drug delivery systems [30], electronic devices [32], biomolecule diagnostic devices [107], chemical sensors [108], and energy applications [51]. Table 3 lists the different properties contributing to the use of diatoms and their frustules in various technological fields. On the other hand, diatoms play a significant role in maintaining water quality [114]. Also, diatoms are the noteworthy algae in the phycoremediation of diverse wastewaters by virtue of their extraordinary cellular machinery. They are experts in utilizing nitrate, phosphate, iron, copper, molybdenum, and silica; in addition, they are capable of remediation of heavy metals like lead, cadmium, chromium, copper, etc. Diatoms show a high degree of flexibility in varied growth conditions that could be useful for their use in challenging conditions [115, 116]. Diatoms are highly sensitive to metal pollution, therefore, diatoms respond quickly to any organic matter or nutrient contamination, for example, metolachlor, atrazine, simazine, phenols, and polyaromatic hydrocarbons [117]. Diatoms have evolved various mechanisms like biotransformation, biomineralization, bioaccumulation, and biosorption to counter heavy metal toxicity [118]. Also, utilizing diatoms as indicators of wastewater quality can be attributed to their presence in diverse ecosystems, their sensitivity to changes in nutrients and environmental conditions, and the ease of access to their diversity [119]. Therefore, diatom [114] was used for nutrient removal as a novel and cost-effective method of water treatment. In addition, the examples of using DB for extraction technique are not numerous, for example, Kirschner et al. [120] used diatomite as a green sorbent for thin-film SPME phase for the isolation and determination of bisphenol A, benzophenone, triclocarban, 4-methylbenzylidene camphor, and 2-ethylhexyl-*p*-methoxycinnamate in environmental water samples. In addition, Reinert et al. [121] used the diatomite as the extraction phase in SPME technique for the determination of polycyclic aromatic hydrocarbons in river water samples by using GC-MS. Moreover, diatomite employed as a biosorbent coating for the determination of methyl paraben, ethyl paraben, benzophenone, and triclocarban in water samples by HPLC-diode array detection (HPLC-DAD) [122]. To the best of our knowledge, there are no examples to use DB for SPE and its miniaturized variant, micro-SPE. Our recent results show that DB can be efficiently used as a stationary phase for backed column capillary LC. Based on these results, we currently work on the utilization of DB in capillary SPE for the isolation of biologically active compounds.

6 | CONCLUSIONS

DB is an interesting material for the development and research, where the three-dimensional structure of DB can be designed as a multifunctional scaffold using various chemical modifications. This article provided a concise overview of the DB regarding the source of origin,

TABLE 3 Characteristics of diatoms which make them proper for different utilities

Applications	Properties	Refs.
Biomedical application (Drug delivery)	• High specific surface area • Thermal stability • Biocompatibility • Customizable surface chemistry • The uniform structure of the nanoscale pore • Inert chemically and biocompatible • Continuous drugs release • Nontoxic	[109]
Bio- and gas sensors	• High surface area • High mechanical resistance • Specific optical characteristics • Biocompatibility	[110]
Electrode material in energy storage applications	• The high theoretical specific capacity • Extremely attractive for energy storage • Extremely attractive for conversion energy	[29]
Nanotechnology and material science	• The cell wall is drenched in pectin and a high volume of silica • Reproducibility of the three-dimensional structures • Ability to self-replicate • Genetic innovation capacity and low production cost • Intricate pore sizes that can be modified • Natural design variability includes costae (rib-like structure plus longitudinal rib and axial rib), canaliculi (channel-like tube), areolae (box-like), punctae (pore like) • For use in boilers and blast furnaces, heat-resistant insulation is favorable • Quite difficult to use as abrasive	[111]
Biosensing	• Large surface area • Optical properties	[112]
Separation science (Stationary phase for thin-layer chromatography) Sample preparation	• The highest void fraction of 96% • Unique nano- and microstructure of the diatom biosilica frustule	[4, 17, 113]

physical-chemical characterization, modification, and application. Biosilica, as a functionalized system, has potential in the field of extraction of bioactive compounds and their separation by using hyphenated analytical techniques. Another area of biosilica utilization, as a porous material, is connected with drug carriers and sensors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study

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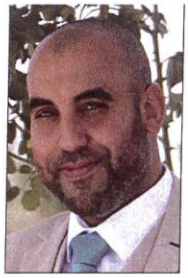
PRZEGLĄD WYBRANYCH METOD ANALITYCZNYCH STOSOWANYCH W ANALIZIE ZANIECZYSZCZEŃ UWALNIANYCH Z MATERIAŁU OPAKOWANIOWEGO | 30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Biokrzemionka, jako niedrogi, przyjazny dla środowiska i łatwo dostępny naturalny biomateriał, uzupełnia technologię syntetycznych mezoporowatych materiałów krzemionkowych.

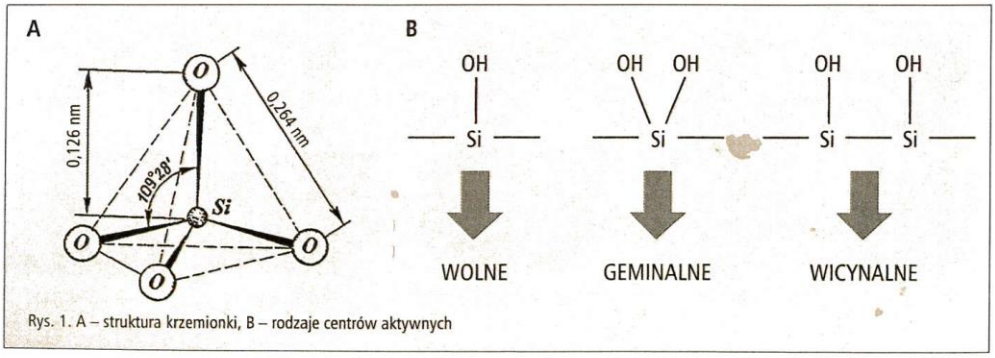
Nowe materiały na bazie 3D biokrzemionki

Rosnące zainteresowanie badawcze wykorzystaniem biokrzemionki okrzemkowej wynika z jej wyjątkowych właściwości, takich jak obojętność chemiczna, biokompatybilność, wysoka stabilność mechaniczna i termiczna, niskie przewodnictwo cieplne, jednorodne struktury porowate o dużej powierzchni właściwej. W przeciwieństwie do produkcji syntetycznych materiałów krzemionkowych o strukturze mikro- lub nanoskalowej w drogim procesie konwencjonalnego wytwarzania biokrzemionkę okrzemkową można produkować w ogromnych ilościach bez znacznego nakładu energii i materiałów. Fakt ten sprawia, iż jest to nieograniczony, łatwo dostępny, naturalny, niedrogi oraz odnawialny materiał. Ponadto, produkcja biokrzemionki jest niezwykle przyjazna dla środowiska, ponieważ zasadniczo nie powstają toksyczne odpady, a proces ten nie wymaga większego zużycia energii w porównaniu z produkcją syntetycznych materiałów na bazie krzemionki. Z tych wszystkich powodów okrzemki są intrygującą alternatywą dla materiałów syntetycznych w opracowywaniu tanich i skutecznych systemów dostarczania leków.

Materiały na bazie krzemionki

Atrakcyjnymi materiałami do obrazowania biomedycznego i zastosowań teranostycznych ze względu na łatwą syntezę, kontrolowaną wielkość cząstek i ładunki powierzchniowe w łagodnych warunkach, opłacalność oraz elastyczną modyfikację przy użyciu różnych grup funkcyjnych i ligandów stanowią mikro/nanocząstki na bazie krzemionki.

Określenie „krzemionka" oznacza substancję o wzorze stechiometrycznym SiO_2, a także jej uwodnioną postać $SiO_2 \cdot nH_2O$. Podstawową jednostką budulcową sieci krzemionki jest anion krzemotlenowy $[SiO_4]^{4-}$, którego modelem przestrzennym jest tetraedr złożony z centralnie położonego jonu krzemu i czterech jonów tlenu O^{2-} ustawionych w narożach (rys. 1A). Kąt pomiędzy wiązaniem -O-Si-O- wynosi 109 °, pomiędzy zaś Si-O-Si zwykle 147 °, jednakże wartość ta może się wahać w granicach od 120 ° do 180 ° w zależności od energii wiązań. Wiązanie pomiędzy tlenem i krzemem Si-O ze względu na mniejszą długość (0,162 nm) niż suma promieni walencyjnych obydwu atomów (0,191 nm) jest najsilniejszym z wiązań tworzo-



Rys. 1. A – struktura krzemionki, B – rodzaje centrów aktywnych

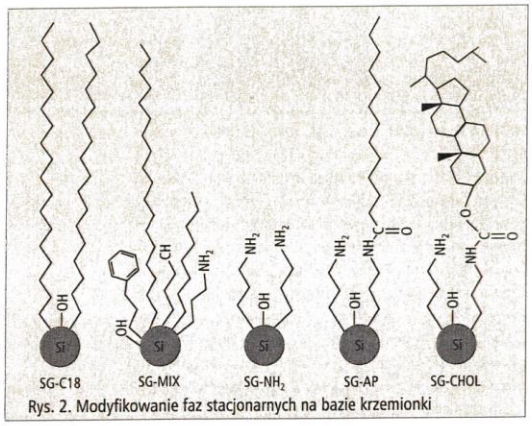
nych przez atom krzemu i odpowiada za jego częściowo jonowy charakter, jak również wysoką stabilność. W zależności od sposobu połączenia tetraedrów sieć krzemionkowa jest najczęściej trójwymiarowa.

Na powierzchni krzemionki występują różne miejsca aktywne (rys. 1B). Są to grupy silanolowe. Grupy te występują jako centra aktywne: wolne (o najwyższej zdolności adsorpcyjnej), geminalne (podwójne) oraz wicynalne. Dodatkowo na powierzchni krzemionki zlokalizowane są grupy siloksanowe, stanowiące 20 % – 35 % w porównaniu z całkowitą liczbą grup silanolowych, które powstają w skutek dehydroksylacji grup hydroksylowych.

Budowa krzemionki sprawia, że jest ona wytrzymała mechanicznie i termicznie. Krzemionka to jedna z najbardziej złożonych i rozpowszechnionych rodzin materiałów. W celu prawidłowej klasyfikacji krzemionki należy uwzględnić cztery podstawowe parametry: strukturę krystaliczną, dyspersję (stała krzemionka zdyspergowana w gazowym lub ciekłym ośrodku dyspersyjnym), porowatość oraz niejednorodność strukturalną i powierzchniową. Ponadto, wyróżniamy również rozpuszczalną krzemionkę, zole krzemionkowe i roztwory spolimeryzowanej krzemionki zbudowane z rozgałęzionych łańcuchów siloksanowych. Krzemionka znajduje zastosowanie w materiałach konstrukcyjnych, mikroelektronice (jako izolator elektryczny) oraz jako komponenty w przemyśle spożywczym i farmaceutycznym. Występuje też w przyrodzie w dużych ilościach i ma dużą zgodność biologiczną. Dodatkowo, jest akceptowana jako materiał „ogólnie uznawany za bezpieczny" (GRAS) przez Amerykańską Agencję ds. Żywności i Leków (FDA) i jest szeroko stosowana w kosmetykach oraz jako dodatek do żywności.

Materiały krzemionkowe mają kilka ważnych właściwości, które czynią z nich unikalną matrycę do wprowadzania funkcjonalnych składników. Po pierwsze, krzemionka jest chemicznie obojętna oraz wykazuje stabilność termiczną i kompatybilność w zastosowaniach biomedycznych (wypełnienia stomatologiczne, kości, stenty). Po drugie, materiały krzemionkowe wykazują znikomą absorpcję w szerokim zakresie długości fal (od ultrafioletu do bliskiej podczerwieni). Pola magnetyczne nie wpływają na nie, co pozwala na elastyczne łączenie różnych elementów bez zmiany odpowiednich właściwości optycznych i/lub magnetycznych.

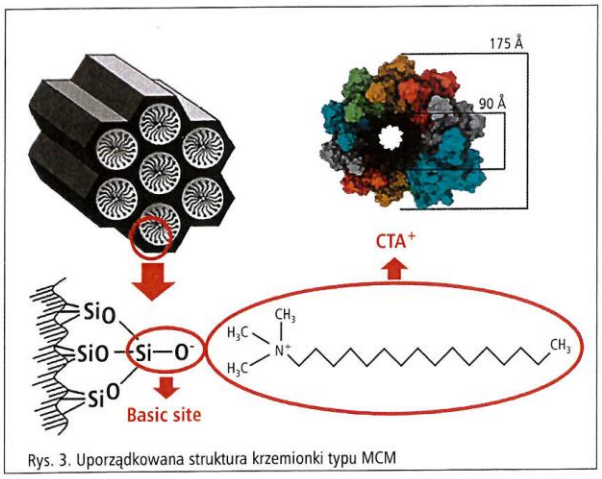
Ponadto, chemiczne modyfikacje powierzchni krzemionki można łatwo przeprowadzić przy użyciu silanowych odczynników sprzęgających, aby umożliwić różnorodne funkcje powierzchni (grupy hydroksylowe/aminowe/tiolowe/karboksylowe), a także ukierunkowaną modyfikację ligandów poprzez funkcjonalizację ugrupowaniami zawierającymi centra hydrofobowe, hydrofilowe, to jest C18, amino, cyjano, fenyl, cholesterol itd. (rys. 2).



Rys. 2. Modyfikowanie faz stacjonarnych na bazie krzemionki

Wśród różnych materiałów porowatych mezoporowata syntetyczna krzemionka jest używana w wielu zastosowaniach dystrybucji leków, w tym w terapii nowotworowej, regeneracji chrząstki, celowanym dostarczaniu leków, poprawie właściwości rozpuszczania leków słabo rozpuszczalnych w wodzie, w preparatach skórnych itp. Takie krzemionkowe materiały mezoporowate, jak na przykład: MCM-41, SBA-15, SBA-16, PHTS, MCF i KIT-6, są szeroko stosowane w dystrybucji (uwalnianiu) leków. Są one pochodzenia naturalnego i biokompatybilne (rys. 3).

Co więcej, są bardzo drogie, czaso- i pracochłonne w syntezie, jak również mogą zawierać pozostałości toksycznych materiałów stosowanych w preparatyce. Alternatywnym podejściem do uzyskania materiałów krzemionkowych z przezwyciężeniem wad i defektów syntetycznych materiałów porowatych jest wykorzystanie naturalnych źródeł krzemionki. Jednym z takich naturalnych źródeł krzemionki są mikroorganizmy, które pobierają, gromadzą i osadzają krzemionkę, tworząc ozdobne i hierarchiczne 3D struktury biokrzemionki.



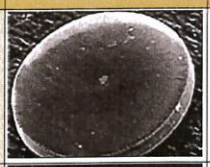
Rys. 3. Uporządkowana struktura krzemionki typu MCM

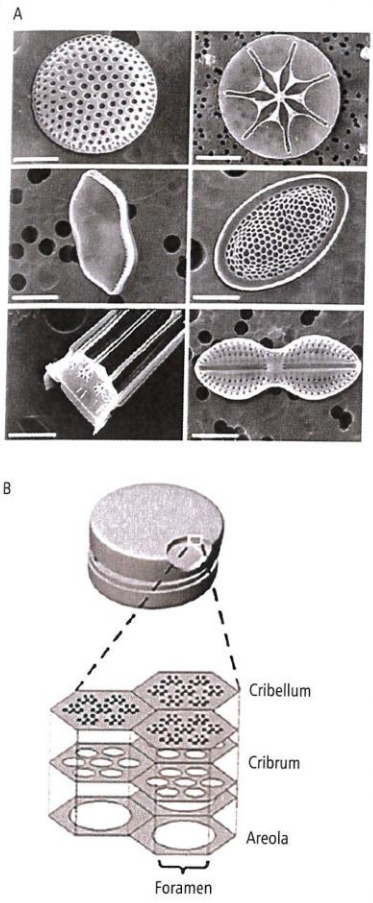
Okrzemki jako naturalne źródła krzemionki

W ostatnim dziesięcioleciu okrzemki są coraz częściej uznawane za obiecujące biomateriały do zastosowań związanych z dostarczaniem leków. Okrzemki mają skomplikowaną, trójwymiarową mikro- lub/i nanoporowatą strukturę o właściwościach fizykochemicznych podobnych do sztucznych krzemionkowych materiałów porowatych. Charakteryzują się hierarchiczną strukturą porów, dużą powierzchnią, wysoką biokompatybilnością oraz cechami optycznymi i fotonicznymi. Pancerzyki okrzemek są wykorzystane jako alternatywa w rozwoju innowacyjnych urządzeń do zastosowań związanych z dostarczaniem leków.

Okrzemki to jednokomórkowe glony występujące w środowisku wodnym na całym świecie. Nazwa grupy pochodzi od charakterystycznych dla ich budowy skorupek krzemionkowych. Ściany komórek okrzemek (zwane pancerzami) są najbardziej imponującym przykładem trójwymiarowej architektury występującej w przyrodzie. Istnieje ponad 200 żywych rodzajów okrzemek, z ponad 100 000 oszacowanych gatunków sklasyfikowanych według ich typowej morfologii i wielko-

Tabela. Średnice przykładowych gatunków okrzemek używanych w dystrybucji leków

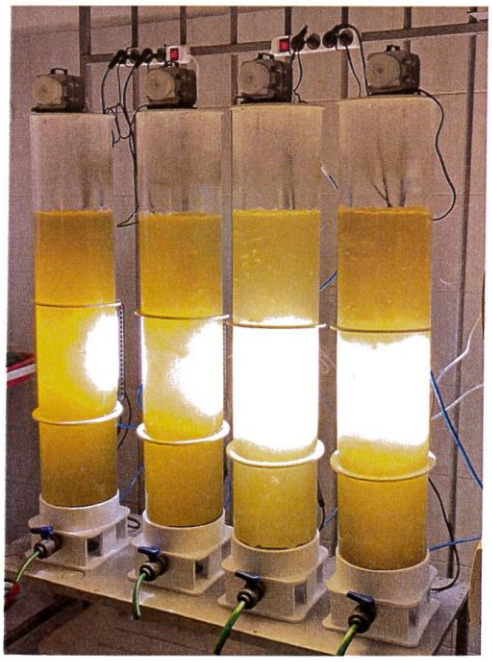
Gatunek okrzemek	Struktura	Średnica
Coscinodiscus wailesii		150 µm
Thalassiosira weissflogii		29 µm
Thalassiosira pseudonana		2 µm
Nitzschia		NA



Rys. 4. A – obrazy ze skaningowego mikroskopu elektronowego (SEM) ścian komórkowych różnych gatunków okrzemek;
B – ilustracja centrycznego pancerza okrzemkowego z profilem przekroju poprzecznego ściany krzemionki, zwykle utworzonej przez trzy nakładające się porowate warstwy: cribellum, cribrum i areola

ści (od 2 µm do 2 mm) (rys. 4A). Systematyka tej grupy opiera się na morfologicznych cechach krzemionkowej skorupki, jej kształcie i urzeźbieniu. Wyróżnia się dwie podklasy: okrzemki promieniste (*Centricae*), mające skorupki o kolistych kształtach, i okrzemki pierzaste (*Pennatae*), charakteryzujące się skorupkami o wydłużonym kształcie. Krzemionkowe pancerzyki okrzemek składają się z dwóch nachodzących na siebie części przypominających dwie szalki Petriego. Górna część nazywana jest wiecz-

kiem (*epitheca*), dolną zaś stanowi denko (*hypotheca*). Wieczko i denko ściśle na siebie zachodzą, jednak nie są ze sobą zrośnięte. Ich górną część stanowi okrywa (*valva*), a ściany boczne tworzą pas obwodowy (*pleura*). Pancerzyk okrzemek stanowi swego rodzaju skomplikowaną rzeźbę, zwaną ornamentacją, na które składają się liczne otworki, żeberka, wstawki itp. Ornamentację okrzemek można obserwować pod mikroskopem świetlnym, występujące na obu stronach okryw charakterystyczne wydrążenia są widoczne pod mikroskopem w postaci prążków. Elektronowa mikroskopia skaningowa umożliwia nam bardziej dokładne i dogłębne obejrzenie struktury pancerzyka. Hierarchiczna organizacja porowatych elementów (np. *cribellum, cribrum, foramen*) obejmuje średnice porów o różnych wzorach, od nanometrów do mikrometrów (rys. 4B). Okrzemki to fascynująca duża grupa glonów, a zmienność morfologii pancerza stwarza naukowcom z różnych dziedzin możliwość doboru gatunków okrzemek do pożądanego zastosowania (tab. 1). Dla przykładu okrzemki z gatunku *Coscinodiscus wailesii* były szeroko badane ze względu na ich zdolność do skupiania światła. Gatunki okrzemek, takie jak *Coscinodiscus concinnus*, *Thalassiosira weissflogii*, *Thalassiosira pseudonana* i *Nitzschia*, zaintrygowały naukowców ze względu na ich zdolność do dostarczania leków, podczas gdy inne gatunki okrzemek o strukturach zawierających pory wielkości nanometrów: *Odontella* i *Phaeodactylum tricornutum*, są uważane za odpowiednie do nanolitografii półprzewodników i spektroskopii fotoluminescencyjnej. Ze względu na swoje właściwości optyczne okrzemki nazywane są niekiedy „klejnotami morza".



Rys. 5. Hodowla okrzemek w fotobioreaktorach w warunkach laboratoryjnych

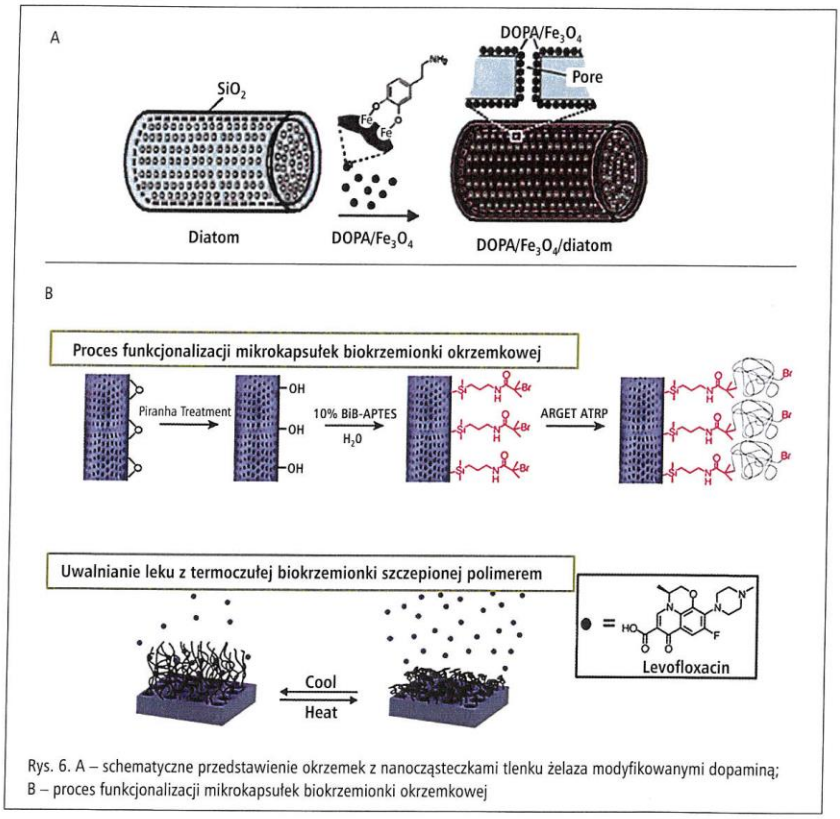
Okrzemki są naturalnym źródłem biokrzemionki i mogą być hodowane w fotobioreaktorach w przyjaznym dla środowiska warunkach i na dużą skalę (rys. 5).

Możliwość ta sprawia, iż krzemionka okrzemkowa jest obiecującą naturalną alternatywą dla syntetycznej krzemionki porowatej w szerokim zakresie biomedycznym, środowiskowym, rolniczym i zastosowaniu energetycznym.

Biokrzemionka jako alternatywa dla syntetycznej krzemionki

Krzemionka okrzemkowa (biokrzemionka) (ang. *diatom biosilica*) to nieorganiczny polimer utworzony przez organizmy, takie jak okrzemki lub gąbki krzemionkowe, z jednostek ortokrzemianowych, w których dwie grupy silanolu są połączone ze sobą jednym wiązaniem siloksanu. Biokrzemionka pochodzi głównie z dwóch źródeł: ziemi okrzemkowej i żywych komórek okrzemek. Diatomit, zwany także ziemią okrzemkową DE (ang. *Diatomaceous Earth*), to materiał kopalny utworzony przez szkielety obumarłych okrzemek, które gromadziły się na dnie jezior lub oceanów przez miliony lat. Ziemia okrzemkowa jest najbardziej rozpowszechnionym źródłem biokrzemionki, szeroko stosowanym jako niedrogi minerał biokrzemionkowy w kilku gałęziach przemysłu (np. w przemyśle spożywczym, rolnictwie, farmacji itp.). Biokrzemionka jest stabilna i nie wykazuje niekontrolowanych reakcji chemicznych podczas mycia, ogrzewania, suszenia czy obróbki enzymatycznej. Wyżej wymienionymi procesami można uzyskać czystą strukturę biokrzemionki pochodzącej z okrzemek. Dlatego jest nietoksycznym i obiecującym biomateriałem w dziedzinie teranostycznej. Pomimo wielkich postępów w dziedzinie nanotechnologii architektura okrzemek może faktycznie konkurować z urządzeniami wytworzonymi przez człowieka. Pomysł wykorzystania pancerzyków krzemionki okrzemkowej w nanotechnologii zaproponowali Gordon i Drum w 1994 roku. Od tego czasu charakterystyczna zdolność okrzemek jednokomórkowych do syntezy biokrzemionki o precyzyjnej 3D strukturze wywołuje rosnące zainteresowanie biologów, chemików i fizyków.

Modyfikacja powierzchni struktur okrzemkowych postępowała w ciągu ostatniej dekady przy użyciu wielu różnych podejść, głównie opartych na strategiach opracowanych dla syntetycznych cząsteczek krzemionki, które obejmują nanoszenie organicznych monowarstw, polimerów, białek oraz powlekanie metalowymi i nieorganicznymi warstwami tlenków. Powierzchnia amorficznej krzemionki wykazuje wysokie poziomy wolnych reaktywnych grup hydroksylowych (–OH), które można wykorzystać do modyfikacji pancerza grupami che-



Rys. 6. A – schematyczne przedstawienie okrzemek z nanocząsteczkami tlenku żelaza modyfikowanymi dopaminą; B – proces funkcjonalizacji mikrokapsułek biokrzemionki okrzemkowej

micznymi (–NH_2, –COOH, –SH i –CHO). Grupy te są odpowiednie do „wiązania" – adsorpcji różnych biocząsteczek z okrzemkami (tj. enzymów, białek, przeciwciał, peptydów, DNA). Dlatego pancerze okrzemek są coraz częściej uznawane za obiecujący materiał w zastosowaniach biomedycznych. Duży stosunek powierzchni do objętości i nanoporowata struktura umożliwiają wiązanie większej ilości leku przekraczający poziomy innych popularnych nośników do dystrybucji leków.

Umieszczenie takich biocząsteczek, jak białka, enzymy czy przeciwciała w strukturze poszczególnych pancerzyków okrzemek, może prowadzić do wytworzenia hybrydowych biosensorów i mikroczipów o wielkości bioreaktorów, które dają nowe możliwości w dziedzinie biotechnologii i nanomedycyny. Istnieją badania dotyczące możliwości zastosowania krzemionki okrzemkowej jako składnika ogniw słonecznych, zastępującej droższy, uczulający dwutlenek tytanu. Inny ekscytujący pomysł dotyczy redukcji krzemionki okrzemkowej do krzemu bez niszczenia unikalnej struktury 3D, co wiązałoby się z nowymi szerokimi możliwościami w dziedzinie mikroelektroniki. Z opublikowanych doniesień wynika, że krzemionkę okrzemkową można modyfikować *in vivo* poprzez izomorficzne podstawianie atomów krzemu w krzemionce w trakcie dodawania do pożywki pierwiastków izomorficznych krzemu (tytanu, germanu).

Funkcjonalizowana biokrzemionka jako nośnik leków

Unikalna porowata struktura, korzystne właściwości mechaniczne, większa przestrzeń wewnętrzna i niski koszt biokrzemionki pochodzącej z okrzemek przyciągnęły badania związane z opracowaniem biokompatybilnej, wysoce przepuszczalnej i nietoksycznej mikrokapsułki do zastosowań związanych z dostarczaniem leków.

Biofunkcjonalizacja (tj. modyfikacja materiału mikro-/nanostrukturalnego w celu dodania funkcji biologicznej) jest jednym z najtrudniejszych i najszybciej rozwijających się elementów dziedziny bioinżynierii, która przekształca obojętne materiały w zaawansowane do konkretnych zastosowań biologicznych.

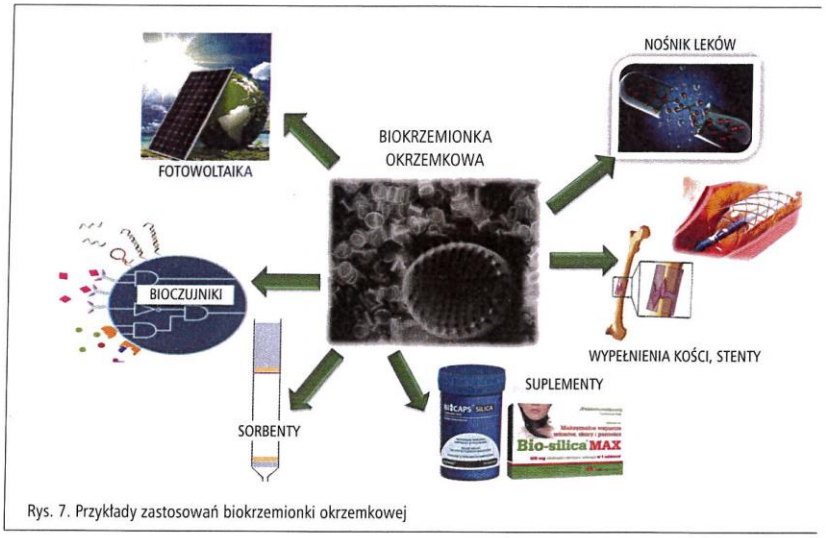
System dostarczania leków oparty na nanomateriałach to nowa metoda dostarczania leków. Nośnik leku to dowolna substancja lub urządzenie używane w proce-

sie dostarczania leku, które służy poprawie selektywności, bezpieczeństwa i skuteczności podawania leku. Jeśli chodzi o skuteczny nośnik leku, musi on nie tylko chronić lek przed degradacją, ale także kontrolować uwalnianie leku do krążenia ogólnoustrojowego w celach terapeutycznych. Nanomateriały zazwyczaj wykazują różne właściwości fizykochemiczne, w tym mały rozmiar, dużą powierzchnię i elastyczną strukturę, co sprawia, że są one potencjalnie interesujące jako nośniki leków. Ponadto, ich nieodłączne właściwości światła, ciepła, magnetyzmu i elektryczności mają ogromne znaczenie dla przygotowania funkcjonalnych i reagujących na bodźce nośników. Wiadomo, że chemiczna modyfikacja porowatych nośników leków jest istotnym czynnikiem kontrolującym dyfuzję i szybkość dostarczania leku. Wiele innych właściwości, takich jak biokompatybilność i wychwyt komórkowy, można poprawić poprzez wiązanie białek lub biomateriałów na powierzchni nośników leków. Modyfikacje powierzchni nie tylko wpływają na interakcje molekularne, ale także kontrolują identyfikację biomolekuł, oferując interesujące perspektywy dla ukierunkowanego transportu leków.

Systemy dostarczania leku o kontrolowanym uwalnianiu mają kilka zalet w porównaniu z konwencjonalnymi postaciami dawkowania o natychmiastowym uwalnianiu, takich jak zmniejszenie częstotliwości dawkowania z poprawą przestrzegania zaleceń przez pacjenta, zmniejszenie całkowitej dawki i skutków ubocznych.

W ostatnich latach zaproponowano kilka strategii wytwarzania, funkcjonalizacji i modyfikacji powierzchni biokrzemionki w celu poprawy zdolności dostarczania leków i kontrolowanego uwalniania leków. Losic i współpracownicy badali funkcjonalizację powierzchni pancerza okrzemkowego i wpływ na charakterystykę sorpcji uwalniania leków nierozpuszczalnych w wodzie. Ze względu na fakt, iż leki hydrofobowe wykazują słabą rozpuszczalność i mogą nie docierać skutecznie do pożądanych miejsc, naukowcy zastosowali biokrzemionkę okrzemkową, aby służyła jako naturalny nośnik leków ułatwiający wiązanie leku. Skonstruowano biokrzemionkę okrzemkową o funkcjonalizowanej powierzchni, zawierającą modyfikowane dopaminą nanocząstki tlenku żelaza obciążone indometacyną, popularnym lekiem przeciwzapalnym, jako nieinwazyjną platformę do ukierunkowanego dostarczania leków (rys. 6A).

Ponadto, ta sama grupa badawcza z powodzeniem udowodniła, że biokrzemionka okrzemkowa z funkcjonalną powierzchnią może służyć jako dobry nośnik leku zarówno do podawania doustnego, jak i implantowanego. Chociaż szczegółowy mechanizm uwalniania leku nie został dokładnie zbadany, w docelowym miejscu wykazano skuteczne wiązanie i uwalnianie dwóch leków, hydrofobowej indometacyny i hydrofilowej gentamycyny, co wskazuje, że naturalna biokrzemionka może potencjalnie zastąpić syntetyczne materiały na bazie krzemionki.



Rys. 7. Przykłady zastosowań biokrzemionki okrzemkowej

Tlenek grafenu (GO) w połączeniu z biokrzemionką został również z powodzeniem zademonstrowany w zastosowaniach związanych z dostarczaniem leków. Zaproponowano nowy materiał nanohybrydowy zależny od pH (biokrzemionka – GO), wykorzystujący strategie wiązania elektrostatycznego i kowalencyjnego. Kowalencyjnie przyłączona biokrzemionka – GO wykazała wyższą zdolność wiązania leku w porównaniu z biokrzemionką – APTES.
Ponadto, Vasani i współpracownicy sfunkcjonalizowali pancerzyki okrzemek w celu skonstruowania reagującego na bodziec nośnika leku (rys. 6B).
Zmodyfikowano powierzchnię krzemionki okrzemkowej za pomocą termoczułych kopolimerów metakrylanu glikolu oligoetylenowego przy użyciu aktywatorów regenerowanych przez polimeryzację rodnikową z przeniesieniem elektronu (ARGET-ATRP), aby umożliwić kontrolowane uwalnianie lewofloksacyny przeciwko dwóm powszechnym patogenom (*Staphylococcus aureus* i *Pseudomonas aeruginosa*) w ranach.
Pomimo że wyprodukowanie syntetycznej krzemionki, takiej jak SBA-15 i MCM-41, jest drogie, pracochłonne i wymaga użycia toksycznych chemikaliów, to systemy te są do tej pory bardzo szeroko stosowane w obszarze dostarczania leków. Jednakże, okrzemki ze względu na naturalną dostępność, nietoksyczność, biokompatybilność, dużą powierzchnię, systematycznie ułożoną strukturę porowatą oraz łatwość modyfikacji powierzchni sprawiły, że są atrakcyjnymi nośnikami leków.

Podsumowanie

Biokrzemionka jest bardzo ważnym środkiem do opracowywania i badania nowych środków terapeutycznych, technik diagnostycznych i nośników leków. Wyniki przeprowadzonych badań wskazują na perspektywy zastosowań przemysłowych biokrzemionki w produkcji środków terapeutycznych. Trójwymiarową strukturę biokrzemionki okrzemkowej można projektować jako wielofunkcyjne rusztowanie za pomocą różnych modyfikacji chemicznych, otwierając drogę do nowej klasy bioinżynieryjnych materiałów mikro- nanostrukturalnych, do zastosowań nie tylko biomedycznych, ale też kosmetycznych, w chemii gospodarczej, fotowoltaice itd. (rys. 7).
Biokrzemionka jako niedrogi, przyjazny dla środowiska i łatwo dostępny naturalny biomateriał uzupełnia technologię syntetycznych mezoporowatych materiałów krzemionkowych. Funkcjonalizacja powierzchni okrzemek stwarza wyjątkową okazję do poprawy ich właściwości fizycznych i chemicznych. Nowym trendem może być projektowanie układów dostarczania leków opartych na biokrzemionce okrzemkowej.

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Diatom biosilica for the chromatographic purposes

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Abstract:	This work presents the use, for the first time, of biosilica in column liquid chromatography. Biosilica is an inorganic polymer consisting of orthosilicate units formed by organisms such as diatoms or siliceous sponges. As diatoms are widespread unicellular photosynthetic algae (Pseudostaurosira trainorii) that produce unique highly ordered siliceous cell walls, called frustules, they can be regarded as a "green" source of silica particles. As the given population of the diatom frustules is characterized by a narrow size distribution and practically the same shape and morphology it was a good rationale to use them in column liquid chromatography. The prepared column filled with siliceous frustules modified with C18 chains showed good chromatographic properties regarding the number of theoretical plates and permeability.
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Toruń, May 6, 2023

Prof. Dr. Marja-Liisa Riekkola
Editor-in-Chief of the Journal of Chromatography A

Dear Professor Riekkola,

Via Editorial Manager we have submitted our article entitled *Diatom biosilica for the chromatographic purposes* written by Michał Szumski, Hussam Al Saoud, Izabela Wojtczak, Myroslav Sprynskyy, Renata Gadzała-Kopciuch, Szymon Bocian, Mikołaj Dembek, Marek Potrzebowski and Bogusław Buszewski. It is a great honor for us to ask you to consider publication of this article in the Journal of Chromatography A.

The proposed article describes preparation and application of diatom siliceous skeletons, that is biosilica, as a new material for high performance liquid chromatography which, as we believe, could be an efficient and "green" chromatographic support. The article contains a Supporting Information showing the comparison of the separation obtained with the described material and a commercially available one.

Sincerely yours,

Michał Szumski

Highlits

Highlights

- Diatom frustules were used as a filling material for liquid chromatographic columns
- Careful preparation of *Pseudostaurosira trainorii* skeletons results in effective HPLC stationary phase
- Column filled with siliceous frustules modified with C18 chains showed good chromatographic properties regarding the number of theoretical plates and permeability

Diatom biosilica for the chromatographic purposes

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Abstract

This work presents the use, for the first time, of biosilica in column liquid chromatography. Biosilica is an inorganic polymer consisting of orthosilicate units formed by organisms such as diatoms or siliceous sponges. As diatoms are widespread unicellular photosynthetic algae (*Pseudostaurosira trainorii)* that produce unique highly ordered siliceous cell walls, called frustules, they can be regarded as a "green" source of silica particles. As the given population of the diatom frustules is characterized by a narrow size distribution and practically the same shape and morphology it was a good rationale to use them in column liquid chromatography. The prepared column filled with siliceous frustules modified with C18 chains showed good chromatographic properties regarding the number of theoretical plates and permeability.

Key words: diatom, biosilica, chromatographic column, high-performance liquid chromatography, stationary phase.

1. Introduction

In liquid chromatography, amorphous silica is a widely used material. This is due to the presence of superficial silanols, which are essential for liquid chromatographic (LC) stationary phase synthesis and column preparation [1]. They provide the hydrophilic nature of silica, which feature is directly used in normal-phase LC, but they also create the possibility of attaching organic moieties to them. The latter approach allows obtaining so-called "chemically bonded phases", which, due to their advantageous properties, have been used in reversed phase and other modes of LC for many years [2,3]. The higher concentration of silanols on the surface makes the silica more hydrophilic in nature. Since surface silanols affect the selectivity of a stationary phase in all LC modes, it is necessary to know their concentration on the surface. This parameter can be determined by several methods like gas chromatography, isotopic substitution, or spectroscopic methods (IR, NMR) [1,4,5,6-10]. Currently, the silica used for LC column packing is synthetic allowing greater control of the final product. The appropriate selection of synthesis parameters ensures obtaining appropriate particle sizes, their spherical shape, porosity and the proper concentration of silanols on the surface. The liquid phase synthesis of silica (silicate hydrolysis) involves the chemical treatment of sand or silica-containing ore to a water-soluble form. Then the formed aggregates of pure and porous silica are washed and dried. Depending on the drying process, a silica gel or powder can be obtained. The properties of the final silica particles are influenced by several parameters, namely temperature, the concentration of the silicate and neutralizing acid solution, pH gradient, ionic strength, final pH, gel aging delay time, effect of additives (e.g. ammonia acts as a coagulant) and mixing energy or other external pressure or other forces applied to the reactor [2,5,11]. Another method of obtaining silica is the gas-phase route to synthesize amorphous silica (pyrogenic silica). Industrial processes involve the oxidation of silicon tetrachloride vapor. This

process occurs in the presence of methane or hydrogen. By modeling the gas composition and combustion speed, silica's particle size and hydrophobicity are optimized. A higher methane or hydrogen content favors the formation of silanols, while a higher oxygen content results in their absence. However, this method is not widely used to obtain silica for LC as the resulting microspheres are non-porous and too small in diameter [2,5].

A natural source of amorphous silica can be, for example, diatomaceous earth or diatomite. Diatomite is a rock that was formed as a result of sedimentation of diatoms which are common unicellular microscopic photosynthesizing algae that possess shells (exoskeletons) called frustules made of amorphous hydrated silica. The sizes of individual diatom cells range from 2 to 200 micrometers. Based on symmetry in frustules morphology, two main groups of diatoms are distinguished: the centric diatoms (mostly discoid shape) with radially symmetrical valve ornamentation and the pennate diatoms (boat-shaped) with bilaterally symmetrical valves. Diatom frustules are composed of two overlapping valves called thecae (upper part – epitheca, lower part - hypotheca) with a characteristic double-sided structure similar to a Petri dish [12–15]. More than 100,000 living diatoms are classified by typical morphologies of their frustules that are unique to each of the diatom species [12]. The frustules forms can be the most diverse and some twisted frustules are regarded as chiral structures [15]. The siliceous walls of diatom frustules that are intricately patterned are decorated by an original pattern of ordered structural features such as pores, ridges, ribs, spikes, or spines, creating the most spectacular example of three-dimensional (3D) structured silica materials of biological origin. The pores of circular, polygonal, or elongated shapes range from a few hundreds of nanometers to a few micrometers in size. The diatom's frustule surface contains a high level of silanol groups (Si-OH) and siloxane bridges (Si-O-Si) [13,15-17]. The intricate design of silica frustules with perfectly ordered hierarchical 3D structures from micro-

73 to nano-scales offered by single-celled diatoms in natural conditions is fascinating for the scientific
74 community [16,18,19]. On one hand the frustules in the form of a natural and processed
75 diatomaceous earth have been used in variety of applications (including chromatography - classical
76 column LC, GC and TLC) for decades, however the form, shape, frustules' integrity and purity
77 was not important parameter. On the other hand, many of the recent studies are focused on
78 utilization of diatoms of a particular species which give a material of high purity characterized by
79 perfectly ordered three-dimensional structure, thermal and mechanical resistance, unique optical
80 properties, and biocompatibility. The modified and unmodified forms of single species diatom
81 biosilica are proposed for applications in the production of optoelectronic devices, biosensors, gas
82 sensors, catalysts, light harvesting, adsorbents, efficient filters, semiconductors, solar cells,
83 templates for nanolithography, carrier drug or building material in the synthesis of bone implants
84 [14,16,17,20-22]. The importance of diatom frustules in the field of modern technologies has
85 become increasingly evident [13-15,23]. Moreover, great possibilities are opened to change the
86 physicochemical properties of the diatom silica by using modification methods for obtaining new
87 silica functional materials with entire frustule shape as well as unique 3D structures preserved
88 [17,24,25]. It is noteworthy here that under given growth conditions (growth medium, temperature,
89 aeration, light conditions), the population of the obtained diatom cells is characterized by a very
90 narrow size distribution and porosity. For such a reason, the diatom frustules would be a promising
91 liquid chromatographic support. As far, only one paper reported the use of single species biosilica
92 as a chromatographic stationary phase in thin layer chromatography [26]. There are no reports of
93 using such a material in column liquid chromatography. The use of biosilica in LC and related
94 techniques (e.g. SPE) would also be potentially promising from the point of view of "green
95 chemistry" as the cultivation of diatoms is rather not environmentally harmful. Taking into account

96 the above, the aim of this work was to investigate the possibility of using diatom frustules as a LC
97 stationary phase.

98 ## 2. Experimental

99 ### 2.1. Materials and equipment

100 Water used as a mobile phase was purified using a Milli-Q system (Millipore, El Paso, TX, USA)
101 in our laboratory. Acetonitrile, methanol, acetone, isopropanol, benzene, toluene, ethylbenzene,
102 propylbenzene and butylbenzene, n-pentylbenzene, thiourea, set of polycyclic aromatic
103 hydrocarbons, paracetamol, carbamazepine, naproxen, diclofenac, ibuprofen, cephalexin and
104 kanamycin were purchased from Sigma Aldrich (St. Louis, MO, USA). Fused silica capillaries
105 with an internal diameter of 400 μm and outer diameter of 1/32" (794 μm) were purchased from
106 Polymicro representative CM Scientific (Silsden, United Kingdom). Kromasil 100 silica gel used
107 in the study was donated by Akzo Nobel (Bohus, Sweden). The 3D-structured diatom biosilica was
108 obtained under laboratory conditions by the cultivation of the selected species of *Pseudostaurosira*
109 *trainorii*. This diatom species was provided by the Culture Collection of Baltic Algae, Institute of
110 Oceanography, University of Gdansk, Poland. Microalgae were cultivated using Erlenmeyer flasks
111 (5 L) with a Guillard's growth medium that contained silicon concentration of 7 mg/L under air
112 aeration and a light regime 12h light/12h darkness. After growing, diatom cells were separated by
113 filtration using a vacuum pump and washed with distilled water. Diatom biosilica (diatom
114 exoskeletons) was isolated from dried diatom biomass by decomposition of the organic matter with
115 hydrogen peroxide and hydrochloric acid. The frustules obtained in this way were washed with
116 distilled water and dried in the oven at 120°C. The physicochemical properties of biosilica used in
117 this research have already been characterized previously by Sprynskyy et al. [27,28]. The
118 chromatographic measurements were performed on a capillary lab-made LC system consisting of
119 Agilent 1260 pump with a 20 μL/min flow regulator, 10-port C-72MX Valco valve with a 200 nL
120 capillary loop, Thermo Crystal-100 UV detector, Jasco FP-2020 Plus fluorescence detector with a
121 4 nL capillary detection cell (Jasco, Japan), and the Clarity chromatography station software (Data
122 Apex Prague, Czech Republic). As the C-72MX Valco valve is designed to be used with 360 μm
123 o.d. fused silica capillaries, the 1/32'' o.d. columns were connected to the injection valve through
124 a 50 mm long piece of the fused silica capillary (75 μm i.d. and 365 μm o.d.) and a P-771 union

125 (IDEX) to provide a zero dead volume connection. The column outlet was connected to the
126 detection capillary (7 cm distance from the connection with the column to the detection window,
127 75 µm i.d. and 365 µm o.d.) via a 2 cm long piece of 1/16'' o.d. FEP tubing whose internal channel
128 diameter was carefully enlarged with a 0.8 mm diameter drill from one side to the half of its length
129 (to accept capillary column outlet) and a 0.4 mm diameter drill from the other side (to accept
130 detection capillary inlet). In this way a tight zero dead volume connection was also provided as the
131 ends of the two capillaries inserted into FEP tubing could touch each other.

132 Micrographs of diatoms and silica gel particles were obtained using a scanning electron microscope
133 with a focused ion beam (SEM/FIB Quanta 200 3D FEG) while EDX spectra were taken with LEO
134 electron microscope model 1430 VP (LEO Electron Microscopy Ltd, United Kingdom). All the
135 experiments were done at the temperature of 22°C.

136 **2.2. Column filling procedure**

137 At first the fused silica capillary section of 3 m in length was consecutively washed with 2 mL of
138 each of the following solvents: dichloromethane, acetone, water, 1M sodium hydroxide, water, and
139 acetone. Then the capillary was cut into 20 cm sections. In each section, the outlet ceramic frit was
140 synthesized using sodium water glass (750 µL) solution in formamide (120 µL), which was
141 introduced by capillary forces to the 2.5 cm distance from the outlet. Then both ends of the capillary
142 were plugged with pieces of silicone rubber and the whole was heated in the oven at 100°C for 1
143 hour. After that such prepared porous frit was flushed with water and acetone and dried in the
144 stream of nitrogen. 50 mg of biosilica was weighed out and suspended in 470 µL of isopropanol.
145 The mixture was then homogenized and degassed by sonication for 10 min. The slurry was
146 transferred to a stainless-steel reservoir (100 x 2 mm) with the empty capillary column connected
147 to its outlet. Such a prepared set-up was connected to a high-pressure air-driven pump (model DSF-
148 122, Haskel, Burbank, USA). The slurry was pushed with methanol at a pressure of 20 MPa for 2
149 hours. After that, the column was left to depressurize slowly. Finally, it was gently disconnected
150 from the reservoir, and the outlet ceramic frit was cut to its final length of 0.5 cm.

151 **2.3. Modification of biosilica surface**

152 Chemical modification of diatom frustules was performed according to the methodology used for
153 silica gel modification described by Buszewski et al. [29,30]. Before chemical modification of the
154 diatom frustules, a sample of the adsorbent was placed in a glass reactor specially designed to
155 protect the reagents from contact with the external environment. Diatom frustules were dried at
156 180°C under vacuum for 24 h to remove physically adsorbed water. The temperature was then
157 reduced to 80°C, and octadecyldimethylchlorosilane dissolved in anhydrous toluene was added. In
158 order to bind hydrogen chloride generated during the reaction, triethylamine was added to the
159 reactor. After 12 h, the reaction product was washed with toluene, ethanol, hexane, and acetone.
160 Then the biosilica was dried in a vacuum oven for 24 h at a temperature of 70°C.

161 **3. Results and discussion**

162 The diatom-based materials have been used in separation sciences for decades and the only form
163 was diatomaceous earth originating from different parts of the world. One of the good definitions
164 that describe this material was presented, for example, by Ergül et al. [31]: "natural diatomaceous
165 earth (DE) is a biogenic sedimentary mineral and originates from the deposition of hard frustules
166 of siliceous algaes (diatoms), which lived in fresh or seawater in the Miocene and Pliocene periods.
167 It is composed of amorphous SiO_2, a variety of inorganic compounds based on metals (such as
168 iron, aluminum, alkali metals, and earth alkaline metals), and a number of organic compounds".
169 The preparation of such a rock for its common uses, like food, cosmetic or chemical industry,
170 filtration etc. comprises such steps as crushing, drying milling and calcination (treatment with high
171 temperature). Although diatomaceous earth can contain more than 90% silica, the above-mentioned
172 impurities are rarely removed during the processing steps. Diatomaceous earth has been used in
173 chromatography for several decades, for example as Chromosorb stationary phase in gas

chromatography or one of the most important support in thin layer chromatography. However, according to our best knowledge it was not used as a packing material for modern LC columns. This might be related to several important reasons: 1) the diatomite itself is a material originating from skeletons of different diatom species so diversity in shape, size, and porosity are natural, 2) taking this into account in connection with the processing steps it is obvious that the particles are not uniform and often crushed, 3) the diatomite originating from different sources (various geographic locations) may differ significantly, 4) the presence of metal (or other elements) impurities may be the cause of undesirable interactions during the separation process. In our approach we decided to use the population of diatom frustules of one selected species (*Pseudostaurosira trainorii*) cultivated and processed under controlled specific conditions to obtain reproducible material of preserved frustules' integrity which could be further modified to obtain a useful liquid chromatographic stationary phase. To compare the material obtained by us and consisting of the frustules only with a typical raw material (unprocessed diatomaceous earth) and a classical diatomite prepared for laboratory purposes (described as "purified and calcined, pure, p.a.") SEM and SEM-EDX measurements were performed. The SEM pictures and corresponding EDX spectra are presented in Figs. S1A, B, C, D and Table 1 in the Supporting Information. It can be clearly seen that the diatomaceous earth (either raw or laboratory grade) contains a significant fraction of debris consisting of some fine material and broken frustules. In contrast, the biosilica which is the subject of this research consists largely of the intact frustules. EDX spectra of the diatomite show the presence (except silicon and oxygen) of such elements as sodium, potassium, calcium, phosphorus, titanium, iron, while in the biosilica some iron is present, but less than in both diatomite samples.

196 The biomass production in our laboratory reactors was ca. 400 mg of dry biomass per liter
197 and the amount of raw frustules we could finally obtain after chemical processing (removal of the
198 biomass) was ca. 20% of the initial biomass. Therefore several cultivation cycles (each lasting
199 about two weeks) were necessary to collect the amount large enough to fill short classical (4.6 or
200 2 mm i.d.) columns (ca. 500 mg of frustules to fill 4.6 mm i.d. column). It also turned out that these
201 classical stainless steel columns turned out to be useless as during the packing process or shortly
202 after we started to use them they were blocked so no flow of the mobile phase could be observed.
203 For these reasons we decided to switch to fused silica capillary columns of 400 μm internal
204 diameter (1/32" that is 794 μm of outer diameter). The advantages of using capillary columns were
205 as follows: small amount of material was needed (50 mg or less) to fill the column, transparent
206 fused silica tubings allowed us to observe the packing process or possible compacting of the bed
207 (in such case the column could be easily shortened to get rid of a dead volume in the column inlet),
208 and with such a relatively large capillary tube which we used, the packing procedure could be easily
209 transferred to normal size HPLC columns in the future.

210 First attempts to fill 400 μm i.d. capillary chromatographic columns with biosilica failed due to the
211 clogging of either the bed that was just built up during the packing process or the ceramic
212 supporting porous frit at the column outlet. We suspected two reasons of these effects: firstly, the
213 residual organic matter (proteins, fat, etc.) insoluble in a packing liquid could be flushed out from
214 the frustules and clog either the bed or a frit, and secondly any fine particles present in a slurry
215 could give the same effect. Sprynskyy et al in their paper mentioned that FTIR spectra of the
216 frustules could suggest the presence of traces of residual fat [16,27]. However, it is rather likely
217 that solvents like chloroform, hexane, methanol, acetonitrile or isopropanol used during the column
218 packing would dissolve the fat matter, so we suspected that some protein residues would clog the

219 column more easily. CP/MAS NMR spectra revealed the presence of the organic matter which is
220 shown in Fig.1A. Therefore, the biosilica was dried in a GC oven for 24 h at a slow temperature
221 gradient from 45 up to 320°C to get rid of any organic content. The NMR spectra did not show the
222 presence of any organic matter after such treatment Fig.1B. Also, it can be concluded from the ^{29}Si
223 CP MAS NMR spectra that the qualitative change in the silica surface structure occurred. A thermal
224 treatment made geminal silanols disappear (Q_2 signal) as well as significant decrease in vicinal and
225 free silanols (Q_3) number with increasing contribution of siloxanes (Q_4) [1,3,31].

226 As for possible fine particulate fraction, we found that it was in fact present in the biosilica sample
227 (see Fig. 2A). The reason for that turned out to be using a magnetic stirrer during the process of
228 wet decomposition of the organic matter with hydrogen peroxide, which could mechanically
229 damage the processed frustules. Having found that we decided rather to use a mechanical stirrer or
230 a shaker to process the diatom biosilica which procedure finally resulted in mostly not broken
231 frustules (see Fig. 2B).

232 The heating of the frustules up to 320°C resulted in another issue. During column packing the flow
233 was frequently blocked again, but this time we saw the reason. In Fig.3 the relatively narrow zones
234 of black matter can be seen in the column inlet. In our opinion, it was a soot generated during the
235 thermal decomposition of the residual organic matter.

236 The SEM/EDX analysis of the frustules taken from this black-colored region has shown 5-6% (per
237 weight) of carbon. According to Rissler et al. [33] the apparent soot density can decrease with
238 particle diameter and may reach such values as 0.15 to 0.53 g/mL depending on the soot source.
239 Such densities are lower than those of organic solvents used, so not surprisingly the soot appeared
240 on top of the column at the end of the packing process. After cutting off the 2 cm section of the

column inlet, which contained the visible black zones, the column became normally permeable. This finding was also a good suggestion that the frustules can be cleaned from soot just by sedimentation and decantation, which in fact worked well. Another way of getting rid of residual organic matter was heating to even higher temperatures like 600°C. Such a treatment did not influence frustules' integrity; however, it was impossible to modify such a material with any chlorosilane. This observation was consistent with earlier thermogravimetric results of Sprynskyy et al. who reported that up to 600°C the biosilica lost its silanol groups [27]. Despite being clean, such a material became rather useless as a potential liquid chromatographic support.

After heating at 320°C, the biosilica was modified with octadecyldimethylchlorosilane to produce an reversed phase (RP) stationary phase. The column prepared with such a material showed surprisingly high efficiencies and we compared it with a column of the same dimensions filled with the commercially available HALO C18 of d_p = 2.7 μm adsorbent. The van Deemter plots of both columns are shown in Fig.4., where *HETP* - height equivalent to a theoretical plate, A - eddy diffusion parameter, $\frac{B}{u}$ - longitudinal diffusion term, and u - mass transfer term.

The plot course stands for relatively good mass transfer across the bed. Plate number as high as 22000 was observed for a 160 mm long biosilica column, which would be comparable to classical spherical silica of around d_p = 3.6 μm. It is known that liquid chromatographic stationary phases can be, in general, divided into several classes: on one hand, taking into account particle shape, 1. irregular particles, 2. spherical particles, 3. monolithic phases can be distinguished, and on the other hand, from the point of view of the particle porosity: 1. non-porous, 2. totally porous and 3. superficially porous (core-shell). However, it is hard to classify a biosilica-based stationary phase as described in this work. On one hand, at the classical approach, such particles should be considered as irregular as they are not spherical, but on the other hand they cannot be considered

as irregular as all of the particles have the same shape, and which is even more important - the same size. Random observations using SEM revealed that the biosilica frustules population which we used is characterized by a narrower size distribution than commercially available Kromasil 100 of d_p = 5 μm. In our current knowledge stage, it is hard to explain such a good chromatographic behavior of the biosilica column. We suspect that the complex influence of the frustules' parameters like valve diameter (ca. 4 μm), its sidewall thickness (ca. 1 μm) and thickness of the bottom (ca. 110-150 nm) in which 150-300 nm pores (holes) are present is responsible for quite good chromatographic properties. Particularly interesting may be the combination of the two last mentioned features. According to the SEM pictures the frustules are distributed across the column randomly. Based on the considerations of Tallarek et al. [34] we can assume that those frustules positioned parallel to the column's long axis would show rather small value of a characteristic length for hydraulic permeability while for those perpendicular ones – this value would equal frustule's diameter. There is, however, a significant difference when compared to spherical particles - the frustules are flat, and in the region of the pores (the holes) their thickness is around 110-150 nm, so perfusion through the frustules is possible. So, even if the frustule material would be totally porous (macroporous), the characteristic length of the stagnant mobile phase would be very short, which would be comparable to superficially porous spherical particles or monolithic silica skeletons [32]. It is very likely that these features are responsible for high efficiency and relatively good permeability. The examined capillary column exhibited a linear pressure *vs* flow rate relationship, with the R^2 = 0.9944, which means that the stationary phase bed was stable within a range of the flow rate used (from 0.75 to 10 μL/min). The permeability of the biosilica and HALO C18 columns (using methanol as a test liquid) were calculated according to Darcy's law Equation 1 and were $K_F = 5.33 \times 10^{-15}$ m^2 and $K_F = 8.74 \times 10^{-15}$ m^2 respectively.

287 $$K_F = \frac{F_m \times \eta \times L}{\Delta P \times \pi \times r^2} \ , \qquad (1)$$

288 where K_F - permeability, F - flow rate, L - column length, η - dynamic viscosity of the fluid ΔP -
289 pressure drop, and r - column radius.

290 The prepared biosilica column was used to separate such test solutes as alkylbenzenes, which can
291 also be useful in assessing the hydrophobicity of the adsorbent used. The mentioned analytes could
292 be separated only at the mobile phase composition 50/50 acetonitrile/water. The obtained peaks
293 are symmetrical and sharp, and no excessive tailing was observed. For a comparison the separation
294 of these test solutes on the HALO C18 column is also presented in Fig. 5; however, at different
295 mobile phase compositions (85/15 acetonitrile/water).

296 The comparison of hydrophobicity of the two columns is shown in Fig.6 by plotting the values of
297 the logarithm of retention factors against the number of carbon atoms in hydrocarbon chains of the
298 alkyl benzene homologs.

299 Much lower hydrophobicity of the biosilica column can be attributed to lower number of accessible
300 surface silanol groups that can be modified, which is a result of much lower surface area of
301 biosilica. However, the coverage density parameter of the biosilica C18 (calculated according to
302 Berendsen-Buszewski equation) has higher value than HALO C18 (4.59 μmol/m^2 versus 2.74 4.59
303 μmol/m^2 respectively) which stands for higher number of accessible silanols. In Fig.7 we present
304 peak data for anthracene obtained on Biosilica C18 and HALO C18 columns. The efficiency and
305 symmetry of this polyaromatic hydrocarbon is much worse on the Biosilica C18 column. This
306 effect may be explained by differences in how alkylbenzenes and polyaromatic hydrocarbons
307 interact with the C18 stationary phases studied. According to the literature data the interactions of
308 alkylbenzenes with C18 ligands occurs mainly via hydrophobic chain-chain interactions which

309 results in sharp peaks of high symmetry on both columns. However, it is likely that polyaromatic
310 hydrocarbons' delocalized π electrons tend to interact with residual silanols present on the biosilica
311 C18 phase which causes poor symmetry (A_s = 1.423) of the anthracene peak [32,35]. On the
312 contrary, good symmetry (A_s = 1.148) and much higher efficiency were observed on the HALO
313 column which contains the end-capped stationary phase. Excessive tailing was also observed
314 during the separation of six PAHs on the Biosilica column, which also contributed to poor
315 separation. Even though the HALO column showed higher retention and efficiency for anthracene
316 it was not possible to baseline separate six polyaromatic hydrocarbons under optimal gradient
317 conditions chosen for either column. A comparison of six PAHs separation chromatograms on
318 both columns is presented in Fig. S2 in the supporting information. Low hydrophobicity of the
319 biosilica C18 column could also be seen in the example separations of anti-inflammatory drugs
320 (small molecules) and FMOC derivatives of two veterinary antibiotics (cephalexin and kanamycin)
321 which are presented in figures S3 and S4 respectively in the supporting information.

322 Nevertheless, in our opinion diatom frustules, after a proper (not very complex, in fact) preparation
323 process may be a promising chromatographic support and further research in this field is to be
324 continued particularly in the field of sample preparation.

325 **4. Conclusions**

326 For the first time diatom biosilica frustules were successfully turned into a useful liquid
327 chromatographic material. Although the frustule-based chromatographic bed did not show
328 selectivity compared to the commercially available adsorbent due to natural porosity and surface
329 properties the observed high efficiencies are very promising. This fact in connection with the
330 availability of such a "green" silica material is, in our opinion, an excellent rationale to continue
331 research in this field.

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Figure captions

Fig.1. NMR spectra for biosilica before (A) and after treatment at 320°C (B).

Fig.2. SEM images for biosilica when a magnetic stirrer (A) or a mechanical stirrer (B) were used during organic matter removal. See text for details.

461 Fig.3. Capillary column with a black matter visible inside the column inlet (A) and a capillary
462 column with clean material after treatment (B).

463 Fig.4. Van Deemter plots for Biosilica C18 *vs* HALO 2.7 µm. Test analytes: thiourea (t_0) and
464 toluene (N). Chromatographic conditions: 50/50 ACN/water, V_{inj} = 0.1 µL, UV detection at λ =
465 200 nm.

466 Fig. 5. Separation of alkylbenzenes (elution order: thiourea (t_0 marker), benzene, toluene,
467 ethylbenzene, propylbenzene, butylbenzene, and n-pentylbenzene) on the A - HALO C18 2.7 µm
468 *vs* B - Biosilica C18 columns. Chromatographic conditions: F = 5 µL/min, for HALO column -
469 ACN/H_2O 85/15, for diatom biosilica C18 column - ACN/H_2O 50/50), UV detection at λ = 200
470 nm.

471 Fig.6. The hydrophobicity comparison of the two columns Biosilica C18 *vs* HALO C18 2.7 µm.
472 Analytical conditions: the same as in Figure 5 except 50/50 ACN/water was used for both columns.

473 Fig.7. Comparison of chromatograms of anthracene obtained on Biosilica C18 and HALO C18
474 capillary columns. Chromatographic conditions F = 5 µL/min, mobile phase ACN/H_2O 50/50, FLD
475 detection at λ_{ex} = 360 nm, λ_{em} = 400 nm.

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Figure 1

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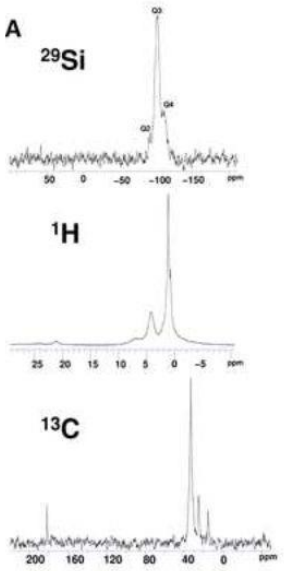

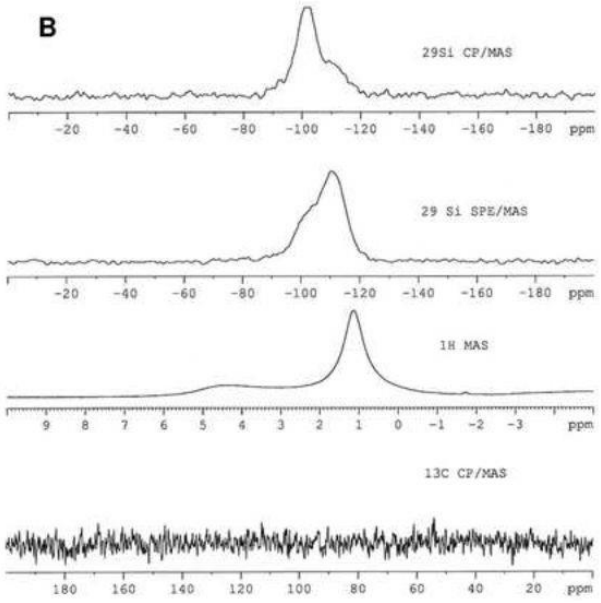

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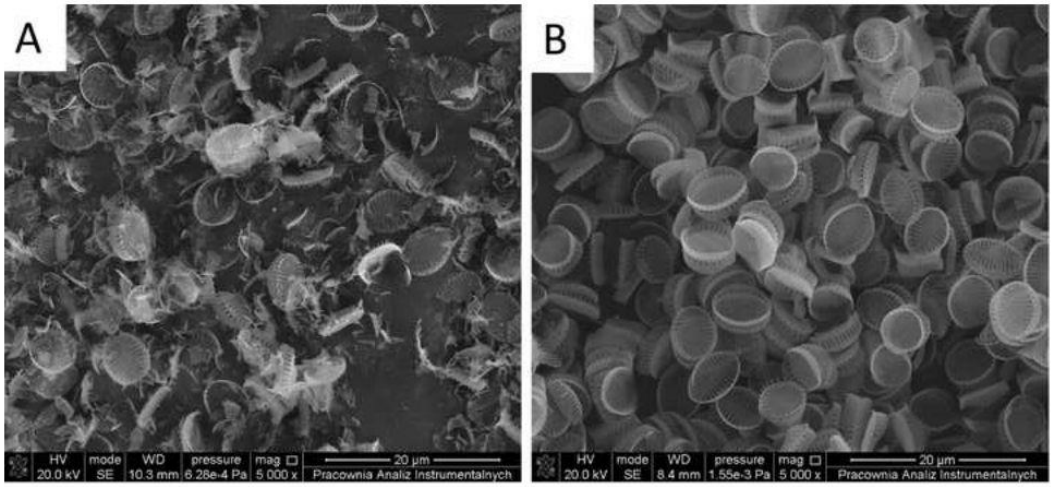

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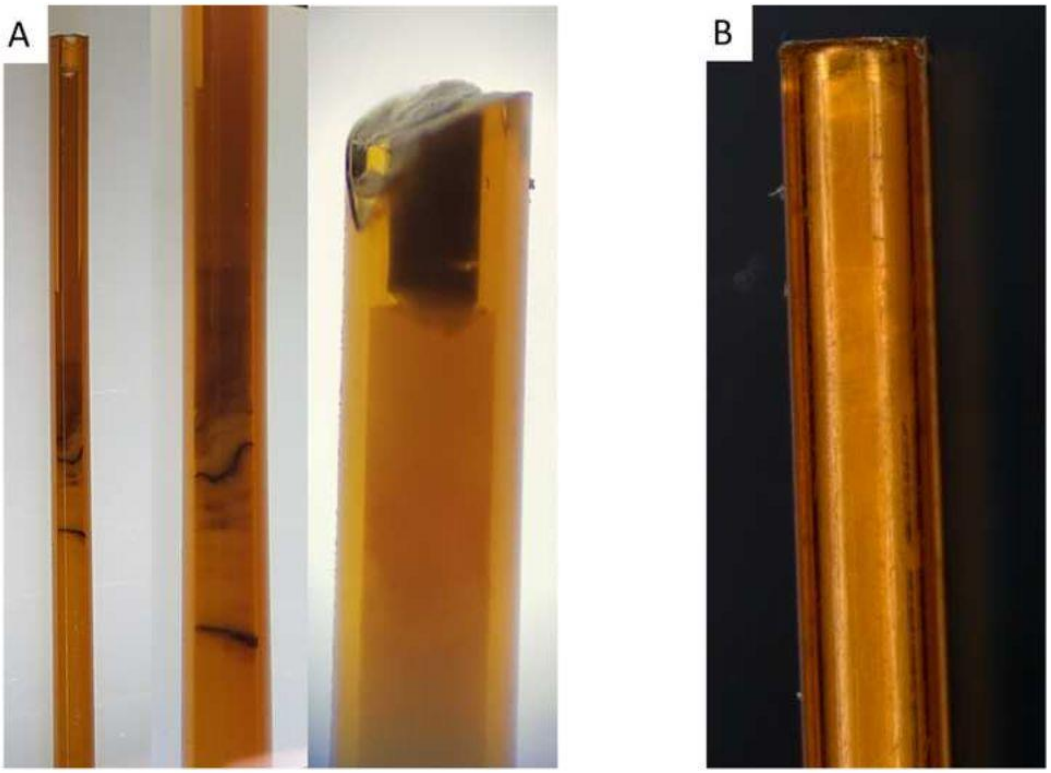

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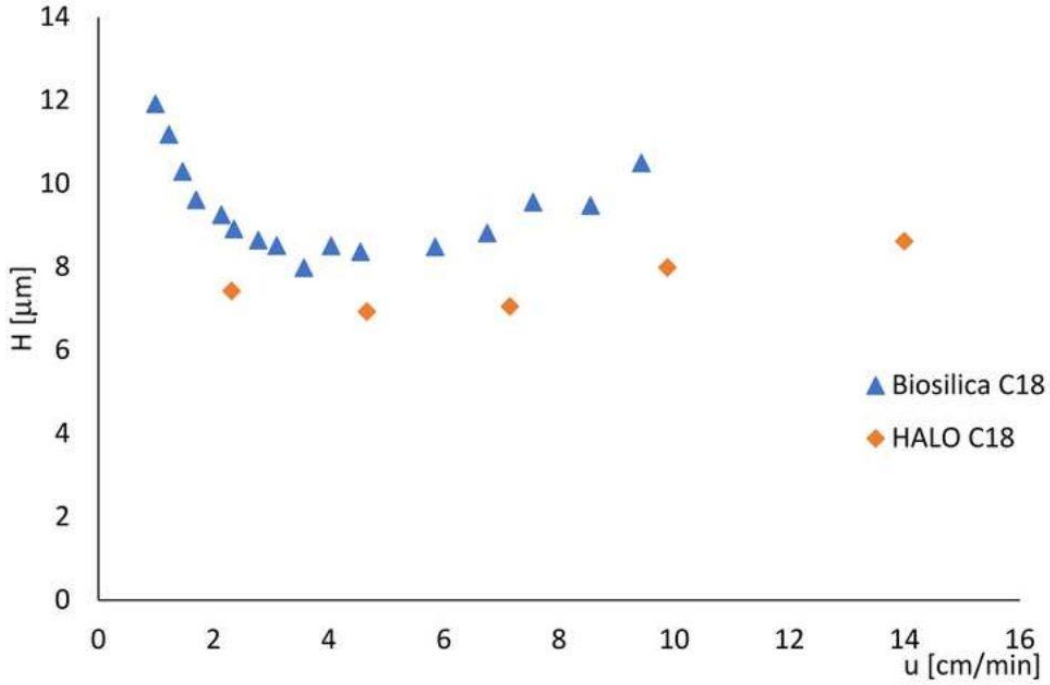

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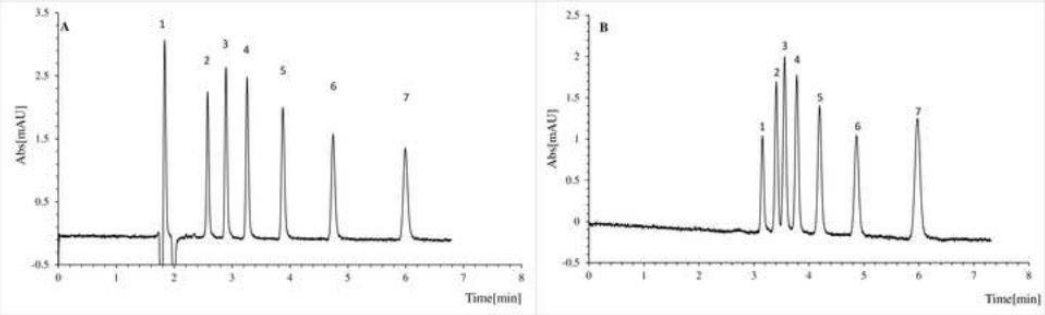

Figure 6

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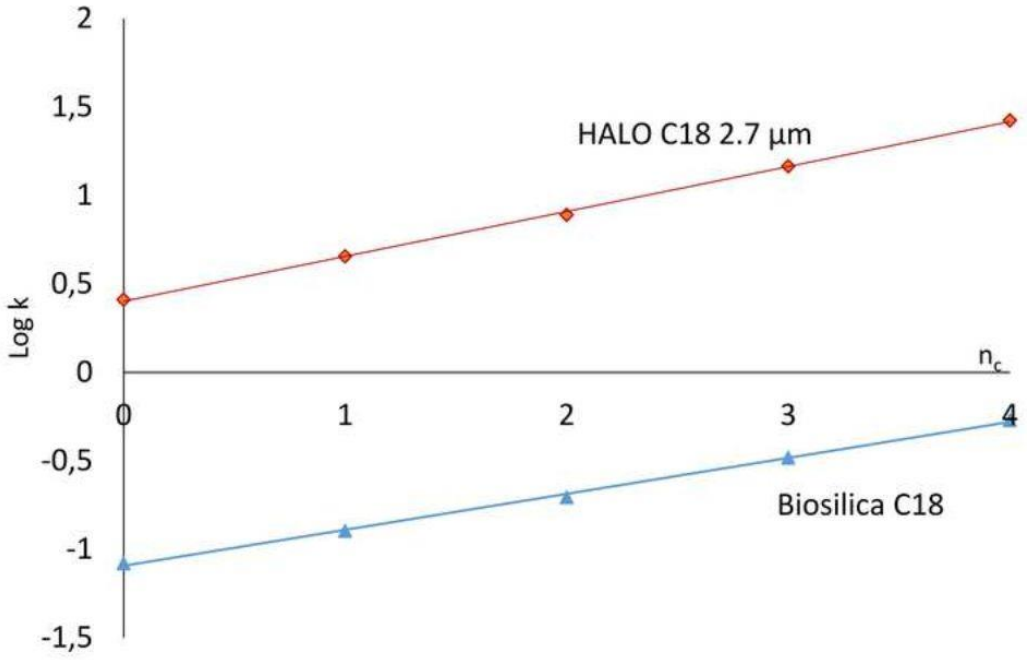

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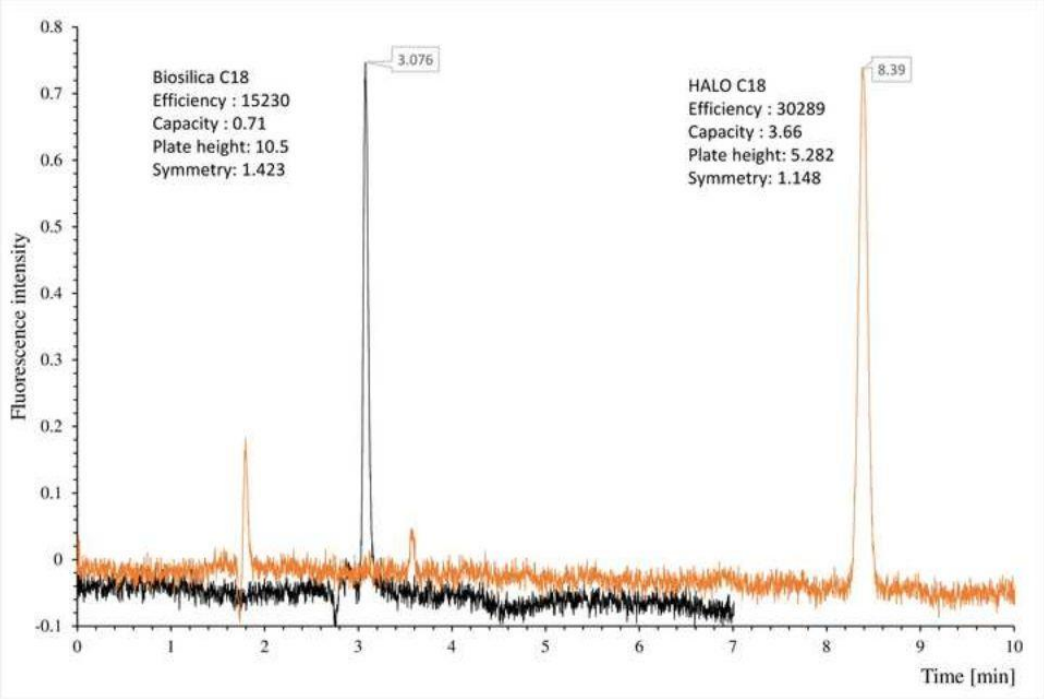

Supporting Info

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Conflict of interest statement

The Authors of the article entitled *Diatom biosilica for the chromatographic purposes* written by Michał Szumski, Hussam Al Saoud, Izabela Wojtczak, Myroslav Sprynskyy, Renata Gadzała-Kopciuch, Szymon Bocian, Mikołaj Dembek, Marek Potrzebowski and Bogusław Buszewski

declare no conflict of interest

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Biosilica as a new stationary phase in HILIC mode

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Abstract

The aim of this work was to use the biosilica obtained from diatoms (microalgae) as a new stationary phase to fill the chromatographic column and test it in high-performance liquid chromatography. Biosilica is an inorganic polymer consisting of orthosilicate units formed by organisms such as diatoms or siliceous sponges. The results showed that the prepared column is characterized by the retention of polar compounds under a high organic solvent content - acetonitrile. As model materials, nucleosides and nucleobases have undergone testing. They were examined separately for retention and attempts to separate test mixtures were successful.

Key words: diatom, biosilica, chromatographic column, hydrophilic interaction liquid chromatography, stationary phase.

Introduction

Hydrophilic interaction liquid chromatography (HILIC) is an alternative high-performance liquid chromatography (HPLC) mode for separating polar compounds. To mention for historical reasons, at first it has been reported that HILIC is a variant of normal-phase liquid chromatography (NPLC), but it turned out that the explanation of HILIC separation mechanism was more complicated than that in NPLC, so this approach was distinguished among LC modes and the acronym HILIC was first suggested by Alpert in 1990 [1]. Compared to traditional normal-phase liquid chromatography and reverse phase liquid chromatography (RPLC), HILIC provides a number of distinct advantages. It is appropriate, for instance, for the analysis of substances in complicated systems that consistently elute close to the void in reserved-phase chromatography. Polar samples exhibit strong solubility in the aqueous mobile phase employed in HILIC, which outweighs the limitations of the frequently encountered poor solubility in NPLC. Since HILIC does not require expensive ion pairing reagents, it is simple to couple it to mass spectrometry (MS), particularly when used in the electrospray ionization (ESI) mode. Gradient elution HILIC, in contrast to RPLC, starts with a low-polarity organic solvent and elutes polar analytes by increasing the polar aqueous concentration [2]. For HILIC separations, any polar chromatographic surface may be utilized. Classical bare silica or silica gels that have been heavily modified with polar functional groups make up the majority of HILIC stationary phases [3]. There is currently an increasing number of references in the literature reporting manufactured or homemade stationary phases that permit working in such environments [4]. For example, amorphous silica is a common form of diatomaceous earth or diatomite in nature. It is a silica-rich substance made from dead diatoms which are microscopic, unicellular photosynthesizing algae with amorphous hydrated silica frustules being their exoskeletons. In addition to silica, diatomaceous earth may also contain trace amounts of iron oxide, clay, and other minerals. It is more frequently utilized in gas chromatography than it is as packing material for LC columns (e.g. Chromosorb stationary phase) [5].

Each diatom cell can be anywhere between 2 and 200 micrometers in size. Diatoms can be divided into two main groups based on the symmetry of their frustule morphology: centric diatoms, which are primarily discoid in shape and have ornamentation on their valves that is symmetrical in all directions, and pennate diatoms, which are boat-shaped and have bilaterally symmetrical valves. Diatom frustules have a distinctive double-sided shape like a Petri dish and are made up of two

overlapping valves called thecae (top part: epitheca, lower part: hypotheca) [6-9]. There are numerous silanol groups (Si-OH) and siloxane bridges (Si-O-Si) on the diatom frustule surface [7,9-10]. Also, there are excellent opportunities to alter the physicochemical characteristics of diatom silica by applying modification techniques to create novel silica functional materials with retained distinctive 3D structures. According to their physicochemical characteristics, diatom frustules also appear to be a potential silica material for chromatographic stationary phase and a packing for liquid chromatographic columns. Therefore, the aim of this work was to prepare and use diatom frustules as a packing for capillary LC columns and test them in a HILIC mode.

Experimental

Materials and equipment

Water used as a mobile phase was purified using a Milli-Q system (Millipore, El Paso, TX, USA) in our laboratory. Acetonitrile, methanol, acetone, isopropanol, adenosine, adenine, cytosine, guanosine, and inosine were purchased from Sigma Aldrich (St. Louis, MO, USA). Fused silica capillaries with an internal diameter of 400 μm and outer diameter of 1/32" (794 μm) were purchased from Polymicro representative CM Scientific Ryefield (EU) Ltd. (Dublin, Ireland). The 3D-structured diatom biosilica was obtained under laboratory conditions by the cultivation of the selected species of *Pseudostaurosira trainorii*. This diatom species was provided by the Culture Collection of Baltic Algae, Institute of Oceanography, University of Gdansk, Poland. Microalgae were cultivated using Erlenmeyer flasks (5 L) with a Guillard's growth medium that contained silicon concentration of 7 mg/L under air aeration and a light regime 12 h light/12 h darkness. After growing, diatom cells were separated by filtration using a vacuum pump and washed with distilled water. At first diatom biosilica (diatom exoskeletons) was isolated from dried diatom biomass by decomposition of the organic matter with hydrogen peroxide and hydrochloric acid followed by washing with water and dried in a vacuum oven at 70°C. After this initial treatment we decided to treat the biosilica with piranha solution (H_2SO_4/H_2O_2 , 3:1) to get rid of some residual organic

matter and increase the number of silanol groups on the surface. The piranha was applied for 40 min then the suspension was washed with distilled water, ethanol, acetone and the resulting material was dried in the vacuum oven at 70°C.

The physicochemical properties of the studied biosilica obtained from *Pseudostaurosira trainorii* have already been characterized previously by Sprynskyy et al. [11,12]. The chromatographic measurements were performed on a capillary lab-made LC system consisting of Agilent 1260 pump with a 20 μL/min flow regulator, 10-port C-72MX Valco valve with a 200 nL capillary loop, Thermo Crystal-100 UV detector, and the PC with the Clarity chromatography station software (Data Apex Prague, Czech Republic). Micrographs of diatoms and silica gel particles were obtained using a scanning electron microscope (SEM/FIB Quanta 200 3D FEG).

Column filling procedure

A 20cm section of the fused silica capillary was consecutively flushed with 2 mL of each of the following solvents: dichloromethane, acetone, water, 1M sodium hydroxide, water, and acetone, and dried in a stream of nitrogen. In each section, the outlet ceramic frit was synthesized using sodium water glass solution (750 μL) in formamide (120 μL), which was introduced by capillary forces to the 2.5 cm distance from the outlet. Then both ends of the capillary were plugged with pieces of silicone rubber and the whole was heated in the oven at 100°C for 1 hour. After that such prepared porous frit was flushed with water and acetone and dried in the stream of nitrogen. 50 mg of biosilica was weighed out and suspended in 470 μL of isopropanol. The mixture was then homogenized and degassed by sonication for 10 min. The slurry was transferred to a stainless-steel reservoir (100 x 2 mm) with the empty capillary column connected to its outlet. Such a prepared set-up was connected to a high-pressure air-driven pump (model DSF-122, Haskel, Burbank, USA). The slurry was pushed with methanol at a pressure of 20 MPa for 2 hours. After that, the column was left to depressurize slowly. Finally, it was gently disconnected from the reservoir, and the outlet ceramic frit was cut to its final length of 0.5 cm.

Results and discussion

SEM imaging

Scanning electron microscopy gave a view of a cleaned diatom frustules' (biosilica) morphological and structural features (biosilica) at different magnifications (Fig. 1). There are clearly visible uniformity and well-preserved forms of the diatom frustules with perforated walls of frustules by a periodic porous network. Diatom frustules can perform an analogous function as porous commercial silica particles. Their porous structure and the presence of polar silanol groups with siloxane result in the retention of polar compounds.

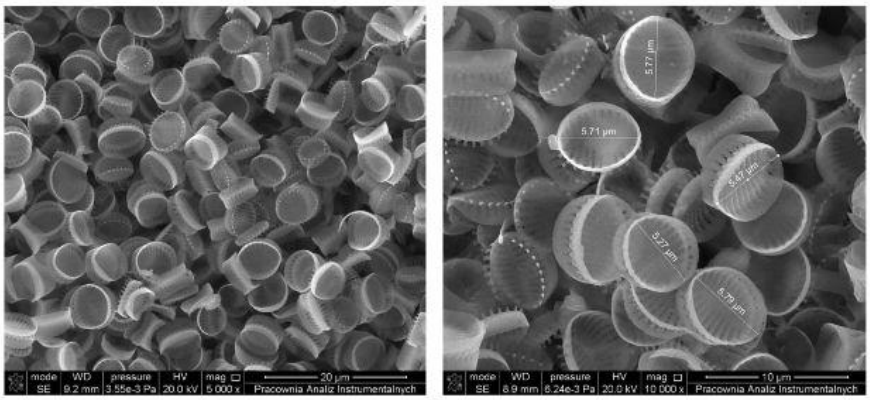

Fig. 1 SEM images of diatom frustules

Chromatographic investigations

The properly prepared diatom material (well preserved frustules without debris) turned out to be a good material for a capillary chromatographic column. The permeability [13] of the biosilica column (using methanol as a test liquid) equaled $K_F = 2.56 \times 10^{-15}$ m^2 and was calculated according to Darcy's law:

$$K_F = \frac{F_m \times \eta \times L}{\Delta P \times \pi \times r^2},$$

where K_F - permeability, F - flow rate, L - column length, η - dynamic viscosity of the fluid, ΔP - pressure drop, and r - column radius.

To confirm diatom biosilica ability to work in HILIC mode, a series of polar compounds were tested belonging to nucleic bases and nucleosides. Five substances were selected for further analysis: adenine, adenosine, guanosine, cytosine, and inosine. First attempts to separate these analytes under isocratic conditions failed and the corresponding retention factors (*k*) as well as peak asymmetry(*f*As) data of all the individually injected analytes are shown in the Table 1. However, we were able to resolve this mixture using a linear gradient. After optimization, a satisfactory separation was achieved with decreasing acetonitrile content in the range 98% - 60%. The optimized gradient was as follows: 98% ACN at 0 – 5 min, next a decrease from 98% to 60% ACN within 20 min. The result of the separation is presented in Fig. 2.

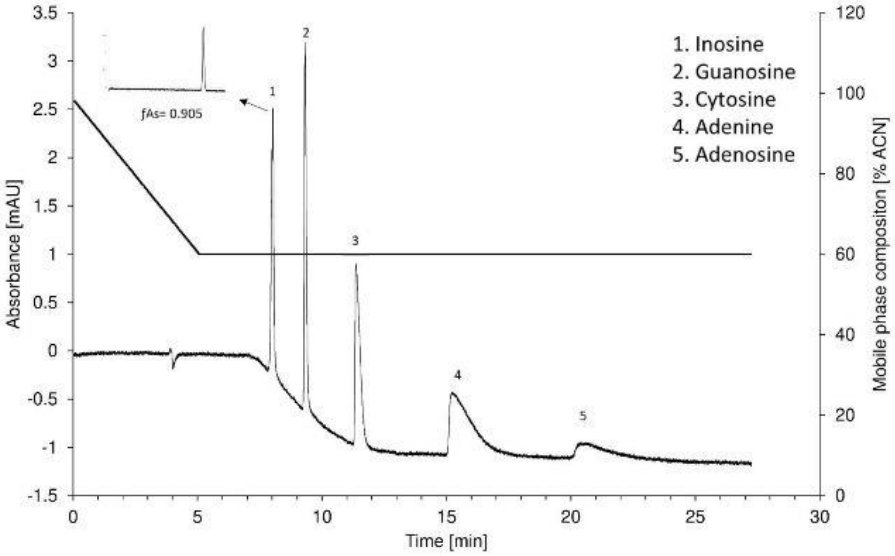

Fig. 2 Separation of mixed nucleic bases and nucleosides (elution order: 1- inosine, 2- guanosine, 3- cytosine, 4- adenine, 5- adenosine). Chromatographic conditions: F = 3 µL/min, ACN/H_2O buffer TFA 0.0001, gradient 0-5 min 98/2 , 5-10 min 60/40, 10-20 min 60/40 , UV detection at λ = 254 nm. Note that the chromatographic system has about 7 minutes of gradient delay resulting from the internal volumes of the pump.

Analyzing the separation results and comparing the gradient elution to the isocratic one, it can be seen that as a result of the change in the composition of the mobile phase with time, the hydro-organic mobile phase forms a water-rich layer on the polar surface of the biosilica/diatoms. The polar analyte interacts not only with silanol groups, but also participates in the interactions of liquid-liquid in the formed layers of mobile phase components [14,15]. The dominant interactions are the hydrogen donors as well as weak electrostatic interactions (with an excess of organic solvent). Hence, such polar individuals as nucleotides will have stronger interactions with the solvated and sorbed near-surface water layer. This contributes not only to the retention of separated analytes, but also to peak symmetry and resolution as we could see in table 1.

Table 1 Characteristic retention data obtained for test compounds data.

Compound	Retention factor (k)/ fAs					
	90/10 ACN/H2O		95/5 ACN/H2O		98/2 CAN/H2O	
Inosine	0.04	1.81	0.24	2.10	2.48	1.27
Guanosine	0.32	2.09	1.86	7.45	3.83	6.02
Cytosine	0.67	4.10	2.21	5.81	4.10	5.34
Adenine	0.77	8.12	2.21	14.76	5.94	9.32
Adenosine	0.68	5.67	3.57	3.90	6.78	1.64

Conclusions

For the first time, diatom biosilica frustules were successfully used as a stationary phase in the HILIC mode. The obtained stationary phase is selective with respect to certain nucleic bases and nucleosides, which were used as test compounds. It was also successful in preparing and optimizing the separation process of a mixture of nucleic bases and nucleosides.

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Author Contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Hussam AL Saoud, Michał Szumski, Sprynskyy Myroslav, Szymon Bocian and Bogusław Buszewski. The first draft of the manuscript was written by Hussam AL Saoud, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability The data that support the findings of this study is available from the corresponding author upon reasonable request.

Declarations

Competing interest The authors declare no competing interests.

Conflict of interest All contributing authors declare no conflict of interest.

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5. CONCLUSIONS AND REMARKS

The principal aim of the present work was to investigate the possibility of using diatom frustules (biosilica) as a liquid chromatography stationary phase, and it would be promising from the point of view of "*green chemistry*" as the cultivation of diatoms is rather not environmentally harmful. The selection of diatom biosilica as a power full material for the development and research, where the three-dimensional structure of diatom biosilica can be designed as a multifunctional scaffold using various chemical modifications.

The results of the study can be summarized as follows:

a) For the first time, diatom biosilica frustules were successfully turned into a useful liquid chromatographic material. Although the frustule-based chromatographic bed did not show selectivity compared to the commercially available adsorbent due to natural porosity and surface properties the observed high efficiencies are very promising.

b) Prepared packing materials based on biosilica were applied for the preparation of columns for nano - HPLC (160 mm, 400 μm i.d) which characterize very high efficiency (*N*) higher than 20000 with low permeability ($K_F = 5.33 \times 10^{-15}$ m^2). The columns have shown long lifetime in analyses of bioactive compounds.

c) For the first time, diatom biosilica frustules were successfully used as a stationary phase in the HILIC mode. The obtained stationary phase is selective with respect to certain nucleic bases and nucleosides, which were used as test compounds. It was also successful in preparing and optimizing the separation process of a mixture of nucleic bases and nucleosides.

d) possibility to use as a material for another application in solid phase extraction for preconcentration and purification of analyte in solid phase extraction (SPE).

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7. ABSTRACT

In recent years, more and more research has focused on the development of new, precisely adaptable, environmentally friendly materials or technologies. Due to the unique properties of the frustule, diatoms are an example of a new natural source of structural biosilica with appropriate properties to produce cost-effective biomaterials. Consequently, in our work we present the use, for the first time, of biosilica in column liquid chromatography. Biosilica is an inorganic polymer consisting of orthosilicate units formed by organisms such as diatoms or siliceous sponges. As diatoms are widespread unicellular photosynthetic algae (*Pseudostaurosira trainorii)* that produce unique highly ordered siliceous cell walls, called frustules, they can be regarded as a "green" source of silica particles. As the given population of the diatom frustules is characterized by a narrow size distribution and practically the same shape and morphology it was a good rationale to use them in column liquid chromatography. The prepared column filled with siliceous frustules modified with C18 chains showed good chromatographic properties regarding the number of theoretical plates and permeability. Also, for the first time, diatom biosilica frustules were successfully used as a stationary phase in the HILIC mode. The obtained stationary phase is selective with respect to certain nucleic bases and nucleosides, which were used as test compounds. It was also successful in preparing and optimizing the separation process of a mixture of nucleic bases and nucleosides.

8. STRESZCZENIE

W ostatnich latach coraz więcej badań skupia się na opracowaniu nowych, precyzyjnie adaptowalnych, przyjaznych środowisku materiałów lub technologii. Ze względu na unikalne właściwości frustuli, okrzemki są przykładem nowego naturalnego źródła biokrzemionki strukturalnej o odpowiednich właściwościach do produkcji opłacalnych biomateriałów. W związku z tym, w naszej pracy przedstawiamy po raz pierwszy zastosowanie biokrzemionki w kolumnowej chromatografii cieczowej. Biokrzemionki to nieorganiczny polimer składający się z jednostek ortokrzemianowych tworzonych przez organizmy takie jak okrzemki czy gąbki krzemionkowe. Ponieważ okrzemki to szeroko rozpowszechnione jednokomórkowe fotosyntetyzujące glony (*Pseudostaurosira trainorii*), które wytwarzają unikalne, wysoce uporządkowane krzemowe ściany komórkowe, zwane frustulami, można je uznać za ,,zielone" źródło cząsteczek krzemionki. Ponieważ dana populacja frustul okrzemek charakteryzuje się wąskim rozkładem wielkości frakcji ziarnowej oraz praktycznie takim samym kształtem i morfologią, było to mocną przesłanką do wykorzystania ich w kolumnowej chromatografii cieczowej. Przygotowana kolumna po odpowiednim oczyszczeniu z depozycji organicznej wypełniona krzemowymi frustulami modyfikowanymi łańcuchami C18 wykazała dobre właściwości chromatograficzne w zakresie liczby płytek teoretycznych i przepuszczalności. Ponadto, po raz pierwszy z powodzeniem zastosowano biokrzemionkowe frustule okrzemek jako fazę stacjonarną w trybie HILIC. Otrzymana faza stacjonarna jest selektywna w stosunku do niektórych zasad nukleinowych i nukleozydów, które zostały użyte jako związki testowe. Udało się również przygotować i zoptymalizować proces rozdzielania mieszaniny zasad nukleinowych i nukleozydów.

9. ACADIMIC ACHEVIMENTS

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3. **Hussam AL Saoud**, Michał Kawalec, Myroslav Sprynskyy, Boguslaw Buszewski. How to Change the World via Science. Diatom biosilica as a drug carrier. poster presentation. Jordan.(09 – 11 June 2022).
4. Boguslaw Buszewski, **Hussam AL Saoud**, Michał Szumski, Myroslav Sprynskyy, Marek Potrzebowski. From Czochralski silica to … biosilica. ^{10}th Congress of Chemical Technology. Oral presentation. Wrocław-Poland.(11-14 May 2022).
5. Michał Szumski, **Hussam AL Saoud**, Myroslav Sprynskyy, Boguslaw Buszewski. Biosilica as a new generation of stationary phase for separation technique. How to Change the World via Science. Oral presentation. Jordan.(09 – 11 June 2022).
6. Boguslaw Buszewski, **Hussam AL Saoud**, Michał Szumski, Szymon Bocian, Myroslav Sprynskyy,. Biosilica as a new generation of material for separation technique. HPLC. Plenary lecture. Duesseldorf, Germany.(2023).

10. STATEMENTS

Hussam AL Saoud, MSc

Chair of Environmental Chemistry & Bioanalytics

Faculty of Chemistry

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7 Gagarina Str., 87-100 Torun, Poland

STATEMENT

I hereby declare that, as co-author of the following publications being a part of my doctoral dissertation, I declare below that my contribution was as follow:

(D1) AL Saoud, H.; Sprynskyy, M.; Pashaei, R.; Kawalec, M.; Pomastowski, P.; Buszewski B. Diatom biosilica: Source, physical-chemical characterization, modification, and application. *J. Sep. Sci*. 2022, 45, 3362-3376.

I have collected the available information about diatom biosilica, source, characterization, modification and functionalization, application from the literatures. In addition, I wrote the initial and the final version of the manuscript. This review article has been written in order to conclude the theoretical part of my PhD dissertation. The figures and tables design were performed by my contribution as well.

(D2) AL Saoud H, Krakowska-Sieprawska A, Sprynskyy M,Pomastowski P, Buszewski B. Nowe materiały na bazie 3D biokrzemionki. Analityka. 2021;3:4–12.

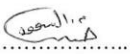
I have collected the available information about diatom biosilica, synthesis, functionalization, from the literature. I wrote the initial and the final version of the manuscript in English.

(D3) M. Szumski, **H. Al Saoud**, I. Wojtczak, M. Sprynskyy, R. Gadzała-Kopciuch, S. Bocian, M. Dembek, M. Potrzebowski, B. Buszewski, Diatom biosilica for the chromatographic purposes. J. Chromatogr. A. (Under review)

I performed sample preparation process, purification of the material, in addition, I have carried out all the analysis using nano-LC, data collecting, Also, I took part of figures and table design and interpretate the results and write the initial and final version of the manuscript.

(D4) AL Saoud, H.; Szumski, M.; Sprynskyy, M.; Bocian, S.; Buszewski, B. Biosilica as a new stationary phase in HILIC mode. Chromatographia. (Under review)

I declare that I have designed the purification of the material. In addition, I have carried out the analysis using nano-LC, data collection and interpreted the obtained data. I declare also that I have written the initial and final version of the manuscript. Figures, table design have also been part of my contribution as well.



.....

Signature

Boguslaw Buszewski

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STATEMENT

I hereby declare, as a co-author of the following publications,

(D1) AL Saoud, H.; Sprynskyy, M.; Pashaei, R.; Kawalec, M.; Pomastowski, P.; **Buszewski B.** Diatom biosilica: Source, physical-chemical characterization, modification, and application. *J. Sep. Sci*. 2022, 45, 3362-3376.

(D2) AL Saoud H, Krakowska-Sieprawska A, Sprynskyy M,Pomastowski P, **Buszewski B.** Nowe materiały na bazie 3D biokrzemionki. Analityka. 2021;3:4-12.

(D3) M. Szumski, H. Al Saoud, I. Wojtczak, M. Sprynskyy, R. Gadzała-Kopciuch, S. Bocian, M. Dembek, M. Potrzebowski, **B. Buszewski**, Diatom biosilica for the chromatographic purposes. J. Chromatogr. A. (Under review).

(D4) AL Saoud, H.; Szumski, M.; Sprynskyy, M.; Bocian, S.; **Buszewski, B.** Biosilica as a new stationary phase in HILIC mode. Chromatographia. (Under review)

Being a part of the doctoral dissertation of Hussam AL Saoud, MSc. I declare that my contribution was to supervise experiments, analysis, and redaction of manuscript. In addition, I managed the projects that supported realization of PhD research.

prof. zw. dr hab. Bogusław Buszewski, dr h.c. mult.

Signature

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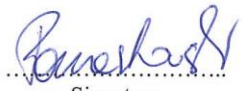
As a co-author of the following publication:

1.**(D1)** AL Saoud, H.; Sprynskyy, M.; Pashaei, R.; Kawalec, M.; **Pomastowski, P.**; Buszewski B. Diatom biosilica: Source, physical-chemical characterization, modification, and application. *J. Sep. Sci.* 2022, 45, 3362-3376.

2. **(D2)** AL Saoud H, Krakowska-Sieprawska A, Sprynskyy M,**Pomastowski P**, Buszewski B. Nowe materiały na bazie 3D biokrzemionki. *Analityka.* 2021;3:4-12.

Which is a part of the doctoral dissertation of Hussam AL Saoud, MSc. I declare that my contribution was to participate in writing part of the abstract in article (D1) and suggestions and co-editing in article (D2).



Signature

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As a co-author of the following publication:

1.**(D1)** AL Saoud, H.; Sprynskyy, M.; Pashaei, R.; **Kawalec, M.**; Pomastowski, P.; Buszewski B. Diatom biosilica: Source, physical-chemical characterization, modification, and application. *J. Sep. Sci*. 2022, 45, 3362-3376.

Which is a part of the doctoral dissertation of MSc. Hussam AL Saoud, I declare that my contribution was to participate in suggestions and co-editing in article (D1)

Michał Kawalec
.....
Signature

Reza Pashaei

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STATEMENT

As a co-author of the following publication:

1.**(D1)** AL Saoud, H.; Sprynskyy, M.; **Pashaei, R.**; Kawalec, M.; Pomastowski, P.; Buszewski B. Diatom biosilica: Source, physical-chemical characterization, modification, and application. *J. Sep. Sci*. 2022, 45, 3362-3376.

Which is a part of the doctoral dissertation of MSc. Hussam AL Saoud, I declare that my contribution was to participate in writing part of the characterization of biosilica (D1).

Signature

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STATEMENT

As a co-author of the following publication:
1.**(D1)** AL Saoud, H.; **Sprynskyy, M.**; Pashaei, R.; Kawalec, M.; Pomastowski, P.; Buszewski B. Diatom biosilica: Source, physical-chemical characterization, modification, and application. *J. Sep. Sci*. 2022, 45, 3362-3376.

2.**(D2)** AL Saoud H, Krakowska-Sieprawska A, **Sprynskyy M**,Pomastowski P, Buszewski B. Nowe materiały na bazie 3D biokrzemionki. Analityka. 2021;3:4-12.

3.(**D3**) M. Szumski, H. Al Saoud, I. Wojtczak, **M. Sprynskyy**, R. Gadzała-Kopciuch, S. Bocian, M. Dembek, M. Potrzebowski, B. Buszewski, Diatom biosilica for the chromatographic purposes. J. Chromatogr. A. (Under review).

4.(**D4**) AL Saoud, H.; Szumski, M.; **Sprynskyy, M**.; Bocian, S.; Buszewski, B. Biosilica as a new stationary phase in HILIC mode. Chromatographia. (Under review).

Which is a part of the doctoral dissertation of Hussam AL Saoud, MSc. I declare that my contribution was to participate in suggestions in articles (D1) and (D2), as well as the conception and development of the methodology of cultivation of diatom material and the initial removal of organic matter (D3) and (D4).

.....
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STATEMENT

As a co-author of the following publication:

1.**(D2)** AL Saoud H, **Krakowska-Sieprawska A**, Sprynskyy M,Pomastowski P, Buszewski B. Nowe materiały na bazie 3D biokrzemionki. Analityka. 2021;3:4-12.

Which is a part of the doctoral dissertation of Hussam AL Saoud, MSc. I declare that my contribution was to participate to write the final version in the Polish language.

Anetta Krakowska-Sieprawska
Signature

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1.(**D3**) **M. Szumski**, H. Al Saoud, I. Wojtczak, M. Sprynskyy, R. Gadzała-Kopciuch, S. Bocian, M. Dembek, M. Potrzebowski, B. Buszewski, Diatom biosilica for the chromatographic purposes. J. Chromatogr. A. (Under review).

2.(**D4**) AL Saoud, H.; **Szumski, M.**; Sprynskyy, M.; Bocian, S.; Buszewski, B. Biosilica as a new stationary phase in HILIC mode. Chromatographia. (Under review)

Which is a part of the doctoral dissertation of Hussam AL Saoud, MSc. I declare that my contribution was to participate in taking part in figures and table design and interpret the results (D3, D4), and take part in writing the initial version of the manuscript in the article (D3) and planning and supervise experiments in both (D3, D4).

Signature

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As a co-author of the following publication:

1. (**D3**) M. Szumski, H. Al Saoud, **I. Wojtczak**, M. Sprynskyy, R. Gadzała-Kopciuch, S. Bocian, M. Dembek, M. Potrzebowski, B. Buszewski, Diatom biosilica for the chromatographic purposes. J. Chromatogr. A. (Under review).

Which is a part of the doctoral dissertation of Hussam AL Saoud, MSc. I declare that my contribution was to participate in the development of the methodology of cultivation of diatom material and the initial removal of organic matter.

Izabela Wojtczak
Signature

Renata Gadzała-Kopciuch
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STATEMENT

As a co-author of the following publication:

1. (**D3**) M. Szumski, H. Al Saoud, I. Wojtczak, M. Sprynskyy, **R. Gadzała-Kopciuch**, S. Bocian, M. Dembek, M. Potrzebowski, B. Buszewski, Diatom biosilica for the chromatographic purposes. J. Chromatogr. A. (Under review).

Which is a part of the doctoral dissertation of Hussam AL Saoud, MSc. I declare that my contribution was to participate in developing the method of purification and fractionation of raw diatom material.

Renata Gadzała-Kopciuch
Signature

Mikołaj Dembek

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STATEMENT

As a co-author of the following publication:

1. (**D3**) M. Szumski, H. Al Saoud, I. Wojtczak, M. Sprynskyy, R. Gadzała-Kopciuch, S. Bocian, **M. Dembek**, M. Potrzebowski, B. Buszewski, Diatom biosilica for the chromatographic purposes. J. Chromatogr. A. (Under review).

Which is a part of the doctoral dissertation of Hussam AL Saoud, MSc. I declare that my contribution was to participate in writing part of the introduction.

Mikołaj Dembek
.....
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Faculty of Chemistry,
Nicolaus Copernicus University in Torun,
Torun, Poland

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STATEMENT

As a co-author of the following publication:

1.(**D3**) M. Szumski, H. Al Saoud, I. Wojtczak, M. Sprynskyy, R. Gadzała-Kopciuch, **S. Bocian**, M. Dembek, M. Potrzebowski, B. Buszewski, Diatom biosilica for the chromatographic purposes. J. Chromatogr. A. (Under review).

2.(**D4**) AL Saoud, H.; Szumski, M.; Sprynskyy, M.; **Bocian, S.**; Buszewski, B. Biosilica as a new stationary phase in HILIC mode. Chromatographia. (Under review)

Which is a part of the doctoral dissertation of Hussam AL Saoud, MSc. I declare that my contribution was to participate in modifying the diatom biosilica in (D3) and taking part in the analysis in (D4).

Szymon Bocian
.....
Signature

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STATEMENT

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1. (**D3**) M. Szumski, H. Al Saoud, I. Wojtczak, M. Sprynskyy, R. Gadzała-Kopciuch, S. Bocian, M. Dembek, **M. Potrzebowski**, B. Buszewski, Diatom biosilica for the chromatographic purposes. J. Chromatogr. A. (Under review).

Which is a part of the doctoral dissertation of Hussam AL Saoud, MSc. I declare that my contribution was to participate in the NMR analysis for the biosilica sample.

……..
Signature