Diatoms – nature materials with great potential for bioapplications

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Abstract

Diatoms are widespread unicellular photosynthetic algae that produce unique highly ordered siliceous cell wall, called frustule. Micro- to nanoporous structure with high surface area that can be easily modified, high mechanical resistance, unique optical features (light focusing and luminescence) and biocompatibility make diatom frustule as a suitable raw material for the development of devices such as bio- and gas sensors, microfluidic particle sorting devices, supercapacitors, batteries, solar cells, electroluminescent devices and drug delivery systems. Their wide availability in the form of fossil remains (diatomite or diatomaceous earth) as well as easy cultivation in the artificial conditions further supports use of diatoms in many different fields of application. This review focused on the recent achievements in the diatom bioapplications such as drug delivery, biomolecules immobilization, bio- and gas sensing, since great progress was made in this field over the last several years.

Keywords: diatoms; drug delivery systems; biosensors, gas sensors; biomolecules immobilization; target drug delivery.

Available online at the Journal website: http://www.ache.org.rs/HI/

REVIEW PAPER

UDC 582.26/.27:66:615

Hem. Ind. **70** (6) 613–627 (2016)

doi: 10.2298/HEMIND150708069M

Diatoms are unicellular, eukaryotic, photosynthetic algae widespread in water habitats [1]. Since their appearance, diatoms have developed into largest group of eukaryotic algae with more than 10^5 estimated species [2]. The facts that they have photosynthetic activity almost equal as all rainforest together and that they produce almost 20 % of the total oxygen and 40 % of the ocean's yearly carbon production illustrate their ecological importance [3]. The whole diatom cell is surrounded with highly ordered 3D porous cell wall made of amorphous silica, which is often denoted as frustule. Diatom frustule is consisted of two overlapping halves called thecae: the upper and larger epitheca and the lower one hypotheca. Several diatom species with different frustule shape and architecture are shown in Figure 1.

Remarkable species specific structure of diatom frustule that is preserved from generation to generation, its biogenesis and possible applications receive special attention during last years especially in the growing field of nanoscience and nanotechnology. Despite rapid technical development, this nature architecture is still superior to all man made devices. Micro- to nanoporous structure with high surface area (up to 200 m^2/g) that can be easily modified through genetic manipulation or chemical reactions, high mechanical resistance, unique optical features (light focusing and luminescence) and biocompatibility make diatom frustule as a useful base in the development of devices, such as devices for water purification and adsorption of heavy metals, bio- and gas sensors, microfluidic particle sorting devices, supercapacitors, batteries, solar cells, electroluminescent devices and drug delivery systems [3–8]. Several excellent reviews focused on elucidation the mechanisms of diatom silica biogenesis have been published in the last few years [5,9–11]. Furthermore, the potential for application of diatoms in chemo- and biosensing, microfluidic devices, drug delivery systems, solar cells, batteries and electroluminescent devices have been also thoroughly reviewed in several papers [3–6,11–14]. Despite the great possibilities for diatom applications, in this review we will focused on the recent development in diatom bioapplications, such as drug delivery, biomolecules immobilization, bio- and gas sensing, due to great progress in this field over the last few years.

Diatoms structure and properties important for bioapplications

During hundred millions years of their existence on the Earth, diatoms have developed some unique features that enable them to survive for such a long time. These features offer us great opportunities for utilization in many areas, either as an alternative to synthetic materials, or for use in areas where synthetic materials are less successful. Diatoms are good example how man, after the era of synthetic materials,

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Paper received: 8 July, 2015
Paper accepted: 20 November, 2015

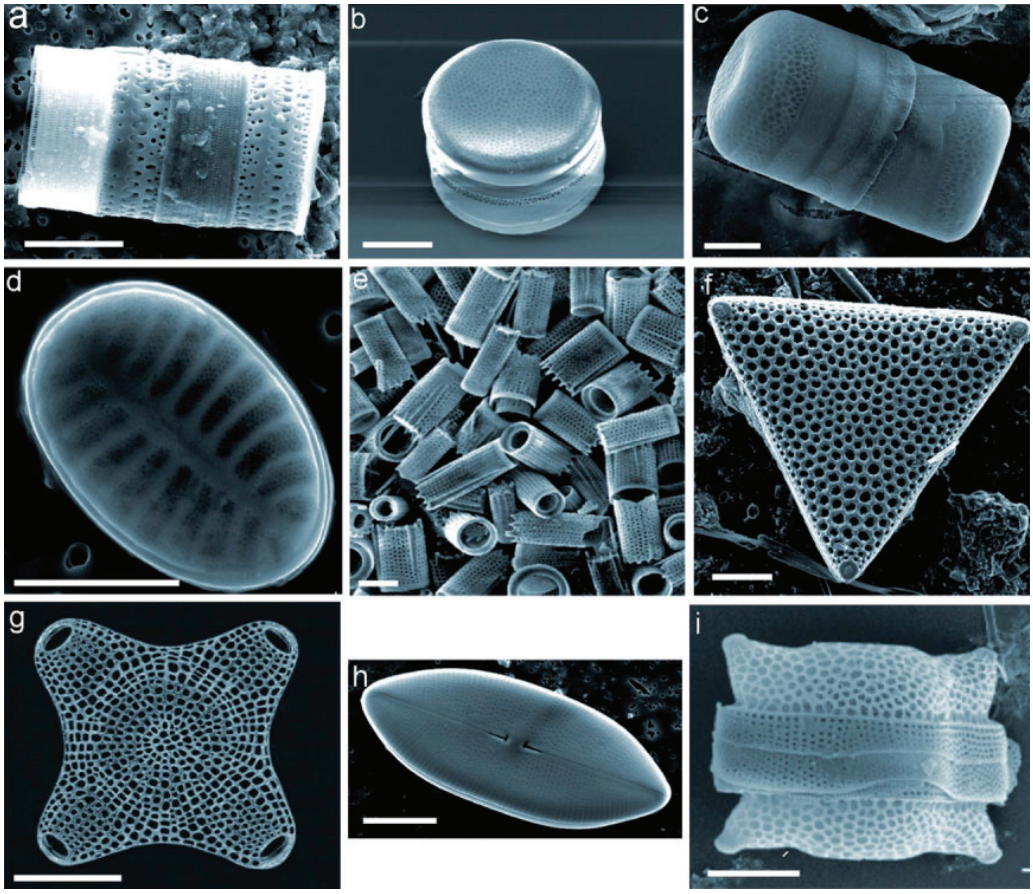

Figure 1. Extraordinary diversity of shapes and structures in diatoms. a–d) and f–i) SEM images of several marine diatom species. e) SEM of fossilized diatom biosilica structures from diatomaceous earth (Diatomite mine NSW, Australia). Scale bar: 10 mm. Reproduced with permission from [6]. Copyright WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 2009.

again returns to nature as a source of cheap materials with unique morphology that can be easily modified through simple chemical reactions. In the following section we will give brief overview of the diatom properties important for their bioapplications.

The most extensively studied diatoms characteristic is, certainly, their intricate cell wall structure. Its 3D architecture, with pores size range from nano- to micrometer size, is precisely reproduced during each cell cycle [5]. While cell size decreases from generation to generation (vegetative reproduction), the size of pores does not scale with the cell size and remains almost constant [14]. Large number of nano- to micrometer-sized pores and presence the silica nanospheres give the frustule high specific surface area and porosity that can be exploited for numerous bioapplications such as: molecular separation, drug delivery, molecule immobilization, bio- and gas sensing. Diatom frustule consists of inorganic (amorphous silica) and organic components (peptides, proteins, glycoproteins and long chain polyamines) [15]. Almost 50 years ago was identified that frustule formation occurs inside membrane bounded organelle, called silica deposition vesicle (SDV) [16]. After formation is complete, silica is deposited on the surface of diatom cell by exocytosis of SDV content. Three groups of organic molecules involved in frustule formation have been identified up to date: silaffin, silacidins and long chain polyamines (LCPAs) [17]. Other identified organic components that are not involved in silica biogenesis contribute to prevention of silica dissolution in the aquatic environment [11,18]. Species specific silica pattern formation probably is determined by specific gene products present inside the SDV, but unfortunately SDV's content hasn't been isolated up to date. So, the current knowledge about mechanisms of frustule morphogenesis is based on the experiments with isolated molecules involved in silica biogenesis [19]. Understanding of the mechanisms of silica biogenesis with recent mapping the genome of two diatom species (*Thalassiosira pseudonana and Phaeodactylum tricornutum*) [11] is of particular importance for development biomedical devices with diatoms. This, altogether with the presence of reactive silanol groups that cover the surface enabling functionalization with different chemical groups give us great opportunities to fine tune physicochemical properties of diatom frustule in order to obtain materials with desired properties for specific bioapplication [3,5].

Diatoms also developed an extraordinary optical features enable them to improve photosynthetic efficiency. De Stefano *et al.* found that diatom *Coscinodiscus wailesii* exhibits the light focusing ability with reducing laser spot size from 100 μm up to less than 10 μm. This phenomenon is explained as the superposition of the waves scattered by the holes present on the surface of the diatom valve [20]. Other interesting diatom optical features are photoluminescence (the excitation of light with a light source) and cathodeluminescence (the excitation of light with an electron beam). Characteristic cathodoluminescence (CL) and photoluminescence (PL) were observed in both culture-grown diatoms and field-collected benthic diatoms, but not in diatomaceous earth (DE) samples. Differences in PL spectra in culture-grown diatoms compared to field-collected benthic diatoms are denoted to more heavily silicified frustule and impurities present in field-collected benthic diatoms that grow in water environment polluted with metals that can possibly influence on frustule architecture and consequently on its luminescence properties [21]. Characteristic diatom PL features are likely to originate from bulk and surface non bridging oxygen absorptions and the radiative decay of self-trapped excitons [21,22]. Characteristic diatoms PL spectra are shown in Figure 2. PL is highly sensitive to the surrounding atmosphere: different substances influence the changes in both intensity and peak position, which can be exploited to obtain dual-parameter optical sensors for high sensitive molecules detection [23]. It is also important to emphasize that PL is surface limited process, *i.e.*, only fraction of molecules adsorbed on the surface could participate in the deexcitations of luminescent centers and influence on PL intensity [24]. Perfect periodic structure offers diatoms capability for coupling incoming light into different waveguide modes and this effect is strongly wavelength dependent [14,25]. Noyes *et al*. found great differences in the intensity of light transmitted through the valve of diatom *C. wailesii* for red, green and blue light. The high intensity was measured for red light-approximately 80% of incoming light intensity, while for blue and green light the transmitted intensity was significantly lower – 20 and 30%, respectively. It is assumed that for utilization of poorly transmitted light (in this case blue and green part of the spectra) diatoms are able to move the chloroplasts close to the cell wall. On the contrary, exploitation of strongly transmitted light (in this case red light) is possible regardless the chloroplasts position related to the cell wall. Strong diffraction was observed when this, long wavelength light was passed through the valve. Light diffraction probably enable uniform distribution of the incoming light in the internal structure of the diatoms and also prevent photobleaching-related damage of chloroplasts when intense illumination is directed on the small surface area [25].

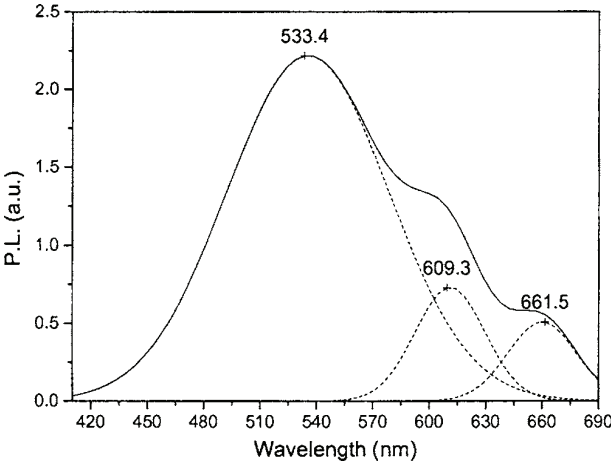

Figure 2. Photoluminescence normalized spectrum of diatoms in air. The dashed lines are the Gaussian profiles corresponding to different bands used to fit the experimental data. The multi-fit procedure has a χ^2 = 0.005. Reprinted with permission from [23]. Copyright AIP Publishing LLC, 2005.

Beside these all diatom features mentioned above, their easy availability and rapid reproduction further encourage their application. Diatoms can grow in artificial condition to more than 10^6 cells per mL of culture medium during only a few days. Their photosynthetic ability and requirements of only light and minimal nutrients make them as a cost effective material desirable for large scale industrial applications [6]. In addition to their feasibility for cultivation, a large and even less expensive source of diatom silica is diatomite or DE, formed from diatom fossils during hundred millions of years [5]. But, it should be noted here, that the properties important for their applications, are not the same amongst fossil and living diatoms. While fossil diatoms exhibit highly condensed and well-organized silica structure, frustules of living diatoms are poorly condensed and less organized. Despite their less organized 3D structure, large number of free silanol groups on the surface of living diatoms gives us greater opportunities for surface functionalization and obtaining advanced materials [26].

Although there are a lot of benefits of using diatoms in constructing biomedical devices, there are also some limitations of their applications. Isolation of diatom frustules from DE can often resulted in obtaining damaged structures, either due to their stay in the earth for thousands of years, or possible damage during mining and purification procedures. Since porous structure and high surface area are the mayor features for diatom bioapplications, physical damage of their structures can seriously limit field of their application. Additionally, diatomite sediments are heterogeneous in nature, containing fossils of different diatom species with different

size and architecture. Since diatom features important for bioapplications are determined by their microstructure, diatomite samples must be undergone to purification and size separation procedures that significantly prolong the whole process. Isolation of diatom frustules from the living organisms requires additional steps of removing organic matter that cover silica structure [4]. Although there are numerous procedures that can be used for purification of raw diatom materials, additional improvements in such procedures are necessary for large scale production processes.

Immobilization of biomolecules into diatom silica

Diatom frustule with high specific surface area and free silanol groups on the surface, high porosity and pore permeability is particularly suitable for immobilization of biomolecules. Although simple protein adsorption on the diatom frustules seems as an easy method, many factors can influence on the amount of adsorbed protein making complex optimization of this process. In the recent study, Lim et al. showed marked differences in protein adsorption capacity between selected centric diatom, pennate diatom and DE samples. They found that protein adsorption on the diatom silica structures is influenced by the surface area, pore volume and surface chemistry of the diatom silica, as well as pH of the protein solution. The dominant adsorption mechanism was chemisorption (hydrogen bonding between hydroxyl group of diatom silica surface and the amino groups or carboxyl groups of proteins), which is desirable, since physisorption can cause protein denaturation [27]. Wang et al. recently investigated the assembling of different kinds of particles (proteins, liposomes and carbon particles) after evaporation onto diatom frustule. They found that the key factor for particle assembly is the large surface area of the frustule, while pore permeability and surface functional groups should be of less importance. This was confirmed with no protein assembling on the carbonyl iron-coated frustule, with remarkably lower surface area [28]. Diatom biosilica particles, modified with 3-aminopropyl triethoxysilane (APTES), and activated with glutaraldehyde, were successfully applied for tyrosinase immobilization. Immobilization of tyrosinase on the diatom biosilica enhanced both its thermal and storage stability compared with free enzyme. Whereas free tyrosinase lost all of its activity during 5 weeks storage at 4 °C in phosphate buffer (0.1 M, pH 7.0), immobilized enzyme lost about 33% of its activity during 8 weeks storage. Lowering of enzyme affinity for the substrate after immobilization is explained by steric hindrance, which limits substrate diffusion to the enzyme active sites. Although immobilization decreased enzyme activity and affinity for the substrate, immobilized tyrosinase showed even higher effectiveness in removing phenol, para-cresol and phenyl acetate during 12 h compared to free enzyme. As immobilized enzymes can be easily separated from the surrounded solution and reused several times, immobilization on the diatom biosilica seems to be promising technique for fabrication of low cost bioreactors for the wastewaters treatment [29].

Isolation of biogenic components responsible for diatom frustule formation opens the doors for *in vitro* protein encapsulation into artificially formed silica structures. However, the main issues that should be considered in this approach are certainly: how to control silica morphogenesis *in vitro* and how to ensure efficient and homogeneous protein entrapment into formed silica structures. During *in vivo* diatom biogenesis, properties of formed silica structures are controlled by posttranslational modifications of proteins involved in biogenesis. Lechner and Becker recently applied chemically modified silaffin peptides, carrying different modifications, which determine morphology of formed silica particles. Covalently linking of target protein to modified silaffin peptides ensured its homogeneous entrapment in the formed silica particles. It is of particular importance that immobilized enzymes retain their activity, compared to free enzymes. If target enzymes were not covalently linked to silaffin peptides, formation of irregular silica structures was observed, with less efficient enzyme encapsulation and lower enzyme activity [30].

Genetic sequencing of two diatom species, *T. pseudonana* and *P. tricornutum*, enabled to immobilize biomolecules, not only by its physicochemical attachment on the diatom frustule surface, but also through genetic engineering [11,31]. This approach, called **li**ving diatom silica immobilization (LiDSI), enables protein immobilization in porous diatom frustules through genetic manipulation of the diatom silica forming machinery [32]. A gene encoding bacterial enzyme hydroxylaminobenzene mutase (HabB) was linked to C terminus of silaffin tpSil3 and incorporated in genome of *T. pseudonana*. During silica biogenesis this fusion protein is incorporated into silica structure and become component of diatom cell wall. Thermostability and stability in organic solvents of HabB was much greater when it was incorporated in diatom biosilica compared with purified HabB in solution. The stability of HabB incorporated in diatoms was equal or even better than HabB encapsulated in silica *in vitro*. Scanning electron microscopy confirmed that genetic manipulation didn't affect silica architecture of *T. pseudonana* [31]. Since HabB is “simple”, monomeric enzyme, recently LiDSI approach was also applied for immobilization of “complex”, oligomeric enzymes that require cofactors and posttranslation modifications for its activity. Successful immobilization of enzymes β-glucuronidase, glucose oxidase, galactose oxidase, and horseradish

peroxidase was achieved in diatom *T. pseudonana*. It is interesting that it is not necessary to add components required for enzyme activity (FAD, heme or Ca^{2+}), since they are incorporated from diatom intracellular compartments [32]. LiDSI approach offers numerous advantages over conventional method of enzyme immobilization, such as physisorption, covalent attachment, or *in situ* incorporation during formation of the synthetic solid material. Since enzyme isolation and purification is often difficult, expensive and could endanger enzyme stability, avoiding these steps is one of the major advantages. The whole process is carried on under physiological conditions, which is desirable for maintaining stability of most of the protein molecules. Diatom frustule with its high mechanical strength, resistance to elevated temperatures, high salt concentration and acidic conditions can extended enzyme shelf-life and possibly prevent enzyme degradation in gastrointestinal tract [31,32]. Beside these undoubted advantages, there are some drawbacks that currently restrain its wide commercial application. The major disadvantage is, certainly, low enzyme loading of only 0.1 mass%, that was achieved in *T. pseudonana*. This is much lower than 0.43 mass%, achieved with enzyme attachment on the surface of *T. pseudonana*. These results are not discouraging, since silica morphogenesis is still not completely explained. It is assumed that a major bottleneck of this process is the capacity for intracellular transport of silaffin-fusion genes to the SDV. With further elucidation of this mechanism, it is likely to be possible to improve enzyme loading capacity [32]. The second disadvantage is variation of enzyme activity amongst different clones due to random integration of the enzyme genes into the diatom genome, resulting in transformants that carry different copy numbers of the transgene within genome regions of different transcriptional activities. On this level of knowledge, numbers of copy and site(s) of transgene integration into diatom genome cannot be controlled [31,32]. LiDSI approach offers us a good feasibility to overcome problems related to delivery of biotechnological drugs such as peptides, proteins, hormones, growth factors, etc. and to enable different routes of their administration, beside the most common parenteral. As number of biotechnological drugs continuously increase, immobilization of biomolecules will certainly be in the focused of further diatom researches.

Diatoms in bio- and gas sensing

Photoluminescent properties of diatom frustules, which are sensitive on surrounding atmosphere, are exploited for highly sensitive gas detection. Electrophilic substances such as nitrogen dioxide, ethanol and acetone have an ability to attract electrons from silica skeleton and consequently quench photoluminescence (Figure 3a) [23]. Beside the changes in PL intensity, the principle peak position is moved to longer wavelengths, due to capillary condensation of the gases in a liquid phase and absorption of volatile substances into the nanometer pores of the diatom frustule, which both increase its average refractive index [22,23]. Nucleophilic substances like pyridine and xylene give free electrons to silica surface and increase the PL intensity (Figure 3b). Effect of both quenching and enhancing PL intensity is completely reversible after changing substances with atmosphere air [23]. Lettieri *et al.* demonstrated high sensitivity of NO_2 detection using high specific surface frustule of diatom *Thalassiosira Rotula Meunier* with detection limit of only 50 ppb [33].

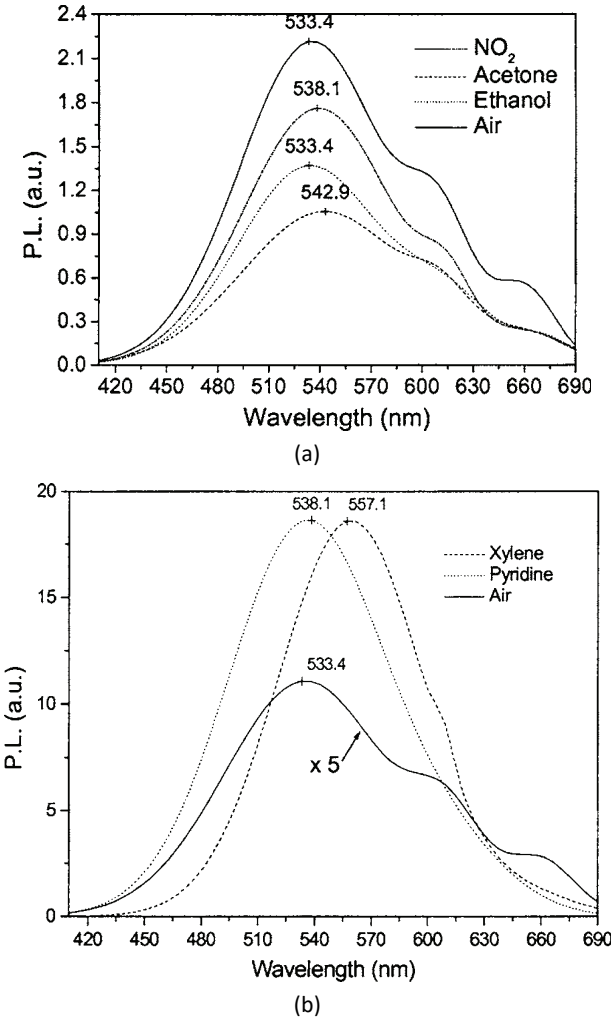

Figure 3. a) Quenching of diatoms photoluminescence spectra in the presence of several gaseous substances: the numbers on top of each spectrum indicate the peak position as estimated by numerical fit. b) Enhancing of diatoms photoluminescence spectra in presence of pyridine and xylene: the numbers on top of each spectrum indicate the peak position as estimated by numerical fit. Reprinted with permission from [23]. Copyright AIP Publishing LLC, 2005.

Despite high sensitivity and ability to distinguish different pure substances, it is not possible to identified

substances in complex mixture based on PL intensity changing in diatoms. Therefore, the chemical modifications of diatom frustule are necessary to obtain biosensors specific for target molecule detection. As mentioned above, the diatom silica surface is particularly suitable for chemical modifications due to presence of highly reactive Si–OH groups. Townley et al. successfully functionalized silica surface of *Coscinodiscus wailesii* with antibodies attached via primary amine group or carbohydrate group. Although both ways are effective, attachment *via* primary amine groups can cause multiple attachment and conformation distortion or undesired attachment at antigen binding sites. In the same study, the diatom frustule was first successfully functionalized with two different antibodies, using mixture of rabbit serum and IgY. Such functionalized diatoms can be used for antibody arrays or immune-precipitation reactions in order to detect presence and quantity of antigen or protein-protein interactions [34]. De Stefano *et al*. designed biosensor capable for specific detection of target molecules in complex mixture based on changing in diatom PL spectra. They attached to diatoms surface monoclonal antibody UN1 that specific interact with G23 protein and this interaction causes enhancing PL intensity and shifting PL peak towards longer wavelengths [35]. Gale *et al.* design biosensor by attachment of rabbit immunoglobulin G (IgG) onto amine functionalized frustule of diatom *Cyclotella sp.* (Figure 4). They found six fold amplification of the frustule PL intensity after IgG attachment and furthermore three times amplification after binding of complementary antigen – anti-rabbit IgG. It was assumed that both antibody and antigen as nucleophilic agents donate electrons to non-radiative defect sites on the diatom frustule and consequently decrease non-radiative electron decay and increase the radiative emission [36].

Lin *et al*. designed a biosensor by integration diatom on the microelectrode platform to detect two inflammatory proteins: C-reactive protein (CRP) and myeloperoxidase (MPO). This sensor is capable for detection antigen-antibody interaction based on changes in electrical properties. When target biomolecule (CRP or MPO) binds to antibodies that are conjugated to the surface of gold microelectrode, perturbation of electrical double layer occurs, resulting in a change in the impedance. Diatom-based biosensors showed superior performance compared with nanoporous alumina membrane-based biosensor and plain metallic thin film biosensor, which is observed based on enhanced response time, dynamic range, signal strength, sensitivity and selectivity. Limit of detection of 1 pg/ml obtained for CRP and MPO is well below the clinically relevant concentrations that makes this sensor useful in identification patients with high cardiovascular risk [37]. Porous silicon surface can also be used for fabrication DNA based biosensors. DNA was bound to surface of the porous silicon microcavities after surface oxidation and subsequent silanization with 3-glycidoxypropyltrimethoxy silane. Such DNA strands are able to recognized and hybridized with complementary sequence. This is utilized for designing viral biosensor for detection bacteriophage lambda virus. DNA hybridization of complementary strands resulted in changing in effective refractive index and consequently 12 nm red shift of the PL peak, that all proved the presence of virus [38].

Frustules functionalized with different biomolecules can be arrayed into portable biosensor devices capable for fast and cheap, routine analyses using small sample amounts and less reagents (lab-on-a-chip-LOP). Rec-

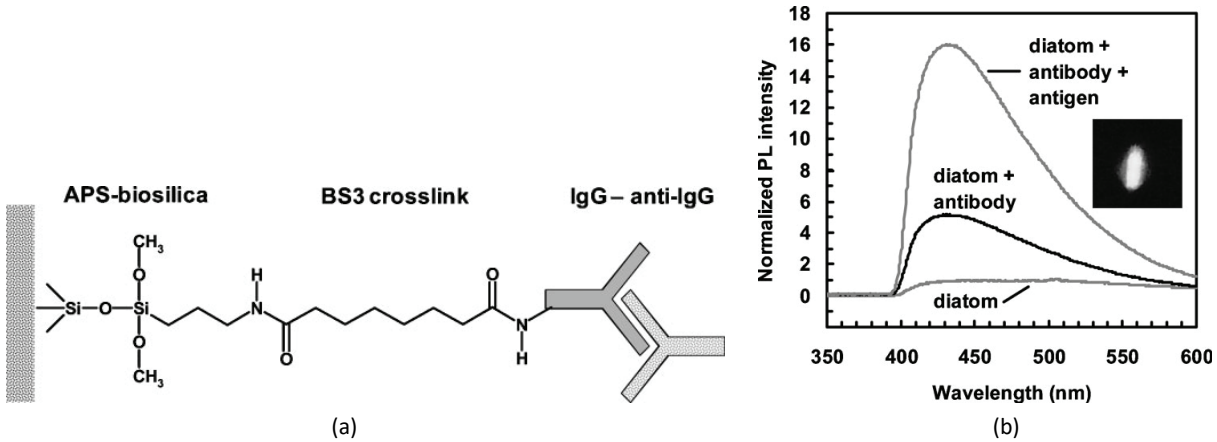

Figure 4. a) Schematic drawing of the structure obtained by covalently atachment of rabbit immunoglobulin G (IgG) onto diatom silica functionalized with 3-aminopropyltrimethoxysilane (BS3-[bis(sulfosuccinimidyl) suberate]-reagent for crossilinking between IgG antibody and amine-functionalized diatom biosilica). (b) Comparison of PL spectra of bare diatom biosilica, rabbit-IgG-antibody--functionalized diatom biosilica (diatom+antibody), and rabbit-IgG-immunocomplex-functionalized diatom biosilica (diatom+antibody+antigen). Excitation wavelength was 337 nm. Inset: photograph of PL emission from antibody-functionalized diatom biosilica. Reproduced with permission from [36]. Copyright WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 2009.

ently, Wang et al. developed biosensor device based on immobilized arrays of diatom frustules functionalized with antibodies. Using diatom frustules, antibodies density was increase, while hybridization time was reduced compared to glass surface. They proposed desired properties of diatoms necessary for optical detection of biomolecules: large surface area, high transparency and pore permeability, smaller sizes and height, and flat surface. As these devices are in the early stage of development, there are still several problems associated with their use. Frustules must be uniformly arrayed with high position precision on the substrate and also uniformly covered with the antibodies. This can be difficult with large frustules, so the use of smaller ones is favoured due to their easy organization into arrays as well as high surface area. The second problem is associated with background interference due to penetration of unbounded antibodies into diatom frustules that influence on luminescence signal. This can be prevented by determination of frustule loading capacity for antibodies followed by their controlled addition until loading capacity is achieved [28]. Diatom based devices demonstrated great potential in highly sensitive detection of gases, biomolecules and viruses. Specific detection of target molecules, the main issue in development of such devices was successfully solved by attachment biomolecular probes that specifically bind to the target ligand. Hierarchical micro- to nanoporous structure further enhances selectivity and sensitivity of diatom based biosensors due to confinement of target molecules into nanoporous structures. This gives an advantage of diatoms over other porous materials in the development of bio- and gas sensors and certainly encourages further studies.

Diatoms as drug delivery carriers

Controlled released drug-delivery systems offer several advantages over the conventional immediate release dosage forms, such as reducing in dosing frequency with improving patient compliance, reduction of total dose and side effects [39]. Due to all these benefits, significant efforts have been made in the development of controlled released drug delivery systems over the past few years. Silica-based mesoporous materials, because of their unique features, such as high surface area, capability for surface modification, order porous structure that enables fine control of drug loading and release kinetics and high pore volume that allows high drug loading capacity, are particularly suitable for drug carriers which can provide drug release over a prolonged period of time [40]. Despite numerous advantages offered by these carriers their synthesis is expensive and requires special experimental conditions and use of toxic solvents that could be retained in final product [41–43].

Highly ordered micro- to nanoporous structure, with large hollow inner space, high specific surface area, low density, high permeability, modifiable surface structure, excellent biocompatibility and low toxicity with their huge availability make diatoms as potential materials in the formulation of different drug delivery systems [44]. Despite these unique features that have been known for many years, application of diatom silica shells in drug delivery is still at the beginning. The main reason for this lie in that DE as the source for isolation of diatom biosilica structures, beside the whole intact silica shells, contains a lot of broken shells, as well as organic and inorganic impurities. Using of such material as drug delivery carrier requires multi step purification procedure that involves removal of impurities from material with the following particle size separation [44].

Application of diatoms in drug delivery was for the first time demonstrated by Losic group [44,45]. In these studies, diatom microcapsules were used as carriers for both hydrophobic (indomethacin) and hydrophilic (gentamicin) drugs. Drug loading capacity of 21.99±2% for indomethacin and 14.5±1% for gentamicin was achieved. It is interesting that during encapsulation crystalline drug is converted into amorphous state and retains in this state during storage period [44,45]. As a pore size is only a few times larger than the drug molecule the formation of crystalline materials is restricted by the confined space of the pores and drug is retained in amorphous state [46]. The amorphous form usually exhibits higher dissolution rate that should improve the bioavailability of poorly soluble drugs, such as the most of drugs today [47]. Characteristic drug release behavior was observed under experimental conditions for testing of implant drug delivery systems: initial burst release of 67–70% of loaded drug in the first 6 h and slow release from 8 h up to complete release of even two weeks [44]. The first phase represent the release of drug molecules physisorbed on the surface and it is unrolled by diffusion mechanism and fits to first order model of release kinetic. The second phase is a consequence of releasing drug encapsulated inside the pores and hydrogen bonded to silanol groups on the surface and it fits to zero order model. Zero order kinetic is desirable for controlled drug delivery system, because the uniform amount of drug is released in each time unit, independent of concentration [44,45]. Mesoporous silica materials exhibit bone regenerative ability when implanted in bone due to depositing calcium and phosphate on its surface and forming apatite layer [48]. This characteristic zero order drug release during almost two weeks [44,45] makes them as promising candidates for implant delivery of antibiotics, anti-inflammatories and bisphosphonates in treatment or prophylaxis of bone infection and osteoporosis which incidence is growing every year. Drug release testing under

experimental conditions for oral dosage forms showed that using of diatom silica microcapsules extended release of indomethacin from 3 to 6–7 h, compared to pure drug [44]. Therefore, using of diatom silica microcapsules seems as a promising approach in the formulation of extended release oral dosage forms. Bariana *et al*. showed marked differences in both drug loading and drug release rate between entire diatom frustules and broken frustules. Sustained drug release and higher encapsulation efficiency can be provided only by using entire diatom frustules [49]. This clearly confirmed that in the development of drug delivery systems with diatoms, particular attention should be paid to purification procedure of raw materials.

Zhang *et al.* investigated the effect of adsorption of prednisone and mesalamine onto diatom silica microshells on drug release behavior as well as permeability through Caco-2/HT-29 co-culture monolayers. They achieved slightly sustained release of both drugs and for the first time demonstrated that diatoms can enhance drug permeability. This beneficial effect of diatoms on drug permeation can be caused by opening the intercellular tight junctions through chelation of Ca^{2+} with negatively charged diatom shells. Reversibility of this effect after incubation of cells in Ca^{2+} medium further supports this hypothesis. Evaluation of toxic potential is essential for each material that is considered for drug delivery applications. Low toxicity of diatoms observed against colon cancer cell lines further supports using of diatoms as drug carriers [50]. In their recent study, Gnanamoorthy *et al*. for the first time demonstrated using of frustules isolated from laboratory cultured marine diatoms as drug delivery carriers. Great potential of isolated frustules as drug carrier was demonstrated with achieved 33% of drug loading for hydrophilic drug streptomycin [51]. Up to now, this is the highest drug loading achieved using diatom frustules as drug carrier, which is comparable or even better compared to synthetic silica carriers. Characteristic biphasic drug release profile was observed, as in the previous papers [44,45], wherein diatom treatment with hydrogen peroxide led to significant changes in drug release profiles. Introduction of surface hydroxyl groups through this treatment procedure extended duration of drug release from 2 days (untreated diatom frustules) to 7 days (diatom frustules treated with hydrogen peroxide), with decreasing drug release in the initial burst stage [51]. Diatom frustules were recently used in the development of drug delivery system intended for improving dissolution rate of poorly soluble drug carbamazepine. Beside carbamazepine adsorption from ethanolic solution onto diatom frustules, completely novel approach was applied which implies adsorption of self-emulsifying suspension with carbamazepine onto diatom frustules that altogether gives solid self-emulsifying drug delivery system (SSEDDS). SSEDDS exhibit superior carbamazepine release rate compared to carbamazepine adsorbed onto diatom frustules from ethanolic solution. Better physicochemical stability was another benefit of diatom SSEDDS over system prepared with simple drug loading from the solution. This was demonstrated with maintaining of the most stable carbamazepine polymorph in the diatom SSEDDS, while in the drug loaded diatom frustules, crystallization of carbamazepine from amorphous form occurred [52]. Due to high specific surface area and great adsorption capacity, diatoms have great potential for using in converting liquid into solid dosage forms, as it was demonstrated in the last mentioned study. This can improve product stability and facilitate production process, but on the other hand often results in increasing formulation bulk beyond acceptable level for oral route of administration. Therefore this approach is suitable only for high potent drugs and further optimization of adsorption process is necessary in order to increase the amount of adsorbed drug and decrease the formulation bulk. Rea *at al.* recently introduced porous diatom nanocarriers, obtained by mechanical crushing followed by particle size separation, as novel carriers for intracellular drug and gene delivery. Reducing in diatom frustules size, from micrometer to nanometer scale facilitated cellular uptake of small interfering ribonucleic acid (siRNA), whose intracellular delivery is difficult due to poor penetration through cell membrane. Obtained diatom nanocarriers conjugated with *siRNA* resulted in efficient cellular uptake of *siRNA* which led to silencing of target *mRNA* genes and consequent down-regulation of target proteins in cancer cells [53].

The first few studies gave promising results regarding the use of diatoms in drug delivery. Encapsulation of drug inside porous diatom silica structure can provide sustained released of drug over several days. However, from the results of few initial studies several limitations of using diatoms as drug delivery carriers have been recognized. The main limitations of these materials are certainly poor drug loading capacity, initial burst drug release due to weak adsorption on the diatom surface and unpredictable drug release, governed by the strength of interactions between drug and diatom silica structures. It is evident that drug loading and drug release rate are markedly influenced with drug chemical structure, due to different interactions with chemical groups on the diatom surface. Therefore, both drug loading and drug release rate can be tune of through chemical modifications of diatom frustules and the results of some recent studies on this topic are mentioned in the text below.

Chemical modifications of diatom frustules for tuning properties important for bioapplications

Despite the great diversity of diatom frustule architecture, numerous modifications were performed to improve its physicochemical properties that are important for their possible bioapplications. It the following part we will mention some of the techniques for modification diatom silica structures with possible applications.

Bao *et al*. converted diatom frustule into co-continuous nanocrystals mixture of silica and magnesia. After selective dissolution of magnesia an interconnected network of silicon nanocrystals which retained starting shape and surface morphology was obtained. The specific surface area of a silicon frustule replica was dramatically increased after magnesia dissolution. Open 3D structure and high specific surface area make this replica suitable for high sensitive gas detection. High sensitive and rapid detection of NO_2, based on changing in impedance, with detection limit of 1 ppm was achieved using sensor that contains this silicon frustule replica. This replica also exhibits photoluminescence after partial oxidation in water [54]. Frustule coating with nanostructured polycrystalline cadmium sulphide (CdS) thin film changed intrinsic diatom photoluminescence depending of the degree of CdS covered surface. After covering less than 25% of the surface, sharper and narrower peaks were observed, which makes this coated frustule more suitable for using in sensor applications. Completely covering with CdS quenches intrinsic photoluminescence and new luminescence signal originates only from deposited CdS film [55]. Losic *et al*. used atomic layer deposition (ALD) technique for reduction pore size of diatom frustule by deposition of ultra-thin layer of titanium oxide (TiO_2) on the frustule of *Coscinodiscus sp.* and *Thalassiosira eccentrica*. ALD provide that TiO_2 is deposited inside the frustule, not only on the surface. The basic pore shape and geometry was preserved after oxide deposition, while pore size was reduced. It is important to emphasize that the reduction in pore size is strongly dependent from number of repeated deposition cycles. Increasing a number of repeated cycles led to greater reduction in pore size. Pore size of only 5 nm should be obtained after 1000 repeated cycles. The precise control of pore size allowed by ALD techniques is necessary for diatom application in molecular separation. TiO_2 coated diatoms can also be used in bio and gas sensing or purification of phosphopeptides. TiO_2 coating also improves mechanical strength and toughness of diatom frustule [56].

Rosi *et al*. functionalized surface of diatom frustule with DNA and used it as a template for sequence-specific assembly of DNA-functionalized gold nanoparticles. Functionalization of surface of diatoms and nanoparticles enabled sequence specific interaction and no assembly was observed when nanoparticles modified with noncomplementary DNA were used. Also, specific multilayer assembly was achieved using successive layers of nanoparticles functionalized with complementary DNA sequences [57].

Although the investigation of diatom applications in drug delivery are still in the early stage, capabilities for tuning both drug loading and drug release rate by surface modification have already been recognized. Magnetically guided drug delivery systems were obtained by functionalization of diatoms with dopamine modified iron-oxide nanoparticles (Figure 5). Beside their potential to be moved under an external magnetic field, dopamine amino groups on the surface are accessible for covalently bonding of biomolecules such as antibodies or DNA. Labeled magnetized diatoms were obtained after immobilization with fluorescein isothiocyanate [58]. Magnetically active diatoms, obtained by frustule modification with iron-oxide nanoparticles can be used as carriers for drug delivery into tumor tissue. This concept was demonstrated with cucrcumin loaded magnetic diatom microshells, prepared by two simple methods. Entrapment into magnetically active diatom microshells significantly improved curcumin cytotoxicity, as demonstrated by reduced cell viability in HeLa cell line. Beside inherent cytotoxicity of iron-oxide nanoparticles, another benefit of magnetically modified diatom microshells is their potential for target drug delivery, guided via external magnetic field. Applied magnetic field can also produce local hyperthermia in tumor tissue, which additionally reduces viability of tumor tissue. It is also possible to modify diatom surface with functional groups that interact with groups in drug molecules.

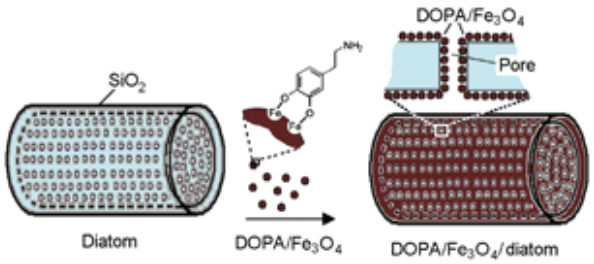

Figure 5. Scheme of functionalization of diatoms with dopamine modified iron oxide nanoparticles to introduce their magnetic properties. Reproduced from [58] with permission of The Royal Society of Chemistry. Copyright The Royal Society of Chemistry, 2010.

As mentioned above, hydrogen bonding slows drug release from diatom microcapsules. Besides hydrogen bonding, electrostatic and hydrophobic interactions should play significant role in both drug loading capacity and release mechanism. Bariana *et al.* functionalized surface of DE microparticles using organo-

silanes and phosphonic acids with either hydrophilic or hydrophobic properties. They found that surface functionalization with hydrophilic groups improves drug loading and provides extended drug release in the case of hydrophobic drug indomethacin. This phenomenon is explained by intermolecular interactions, such as Van der Waals and electrostatic interactions, between drug molecules and polar functional groups on the modified DE surface. On the other side, functionalization with hydrophobic groups resulted in lower indomethacin loading with more pronounced initial burst release. It is assumed that long carbon chains on the DE surface form barrier which hinders permeation of drug molecules into porous DE structure and also decreases drug loading on the surface. Completely opposite situation is observed with hydrophilic drug gentamicin. In this case extended release and higher drug loading is achieved with hydrophobic surface functionalization. DE surface modification with hydrophilic groups led to lower gentamicin loading and faster release rate due to ionic repulsion forces between groups of same charge in both drug molecule and DE surface [59]. Recently, application of diatom biosilica modified with graphene as drug delivery carrier was demonstrated. Graphene, due its outstanding properties, is recognized as material of 21st century which discovery in 2005 was awarded by Nobel price [60]. In this paper two different approaches for the preparation diatom–graphene hybrids based on covalent coupling of graphen oxide (GO) sheets onto the amine terminal groups of 3-aminopropyltriethoxysilane (APTES) functionalized diatom surface and electrostatic attachment on positively charged APTES functionalized DE particles were demonstrated (Figure 6). These GO decorated DE nano-hybrids were used as smart pH sensitive micro drug carriers at pH 7.4 and pH 3.5 for model drug, indomethacin (IMC). Obtained DE-GO nano-hybrid materials exhibited significantly higher drug loading, with pH sensitive drug release and decreased initial burst drug release compared to APTES functionalized DE particles without attached GO. Observed phenomena are consequence of increased interactions between drug and GO attached to DE surface, particularly hydrogen bonds which are sensitive on pH changes [61].

Further studies in the field of functionalized diatom-based drug or gene delivery systems should be focused on the development of so called stimuli responsive drug delivery systems that were successfully developed numerous times with synthetic mesoporous silica carriers. In these drug delivery systems attachment of different ligands closes the pores and prevents drug release until specific internal (changing in pH or temperature, different chemical or enzyme reactions), or external (UV radiation, magnetic or electric field) stimulus occurred. This concept is particularly important in the case of cytotoxic drugs, where it is essential to prevent premature drug release and toxic effects on health tissues. Vasani *et al*. recently for the first time demonstrated development of diatom based stimuli responsive drug delivery systems. They employed surface-initiated activators regenerated by electron transfer based atom transfer radical polymerisation to graft thermo-responsive copolymers of ethylene glycol methacrylates to the surface of diatom biosilica microcapsules (Figure 7). The application of the resulting composites for thermo-responsive drug delivery of levofloxacin with strong temperature dependence was demonstrated. Levofloxacin release profiles from developed thermo-responsive drug delivery systems are presented on Figure 8 [62]. One of the main benefits of such system is that thermo-responsive polymer act as physical barrier for drug release and there are no

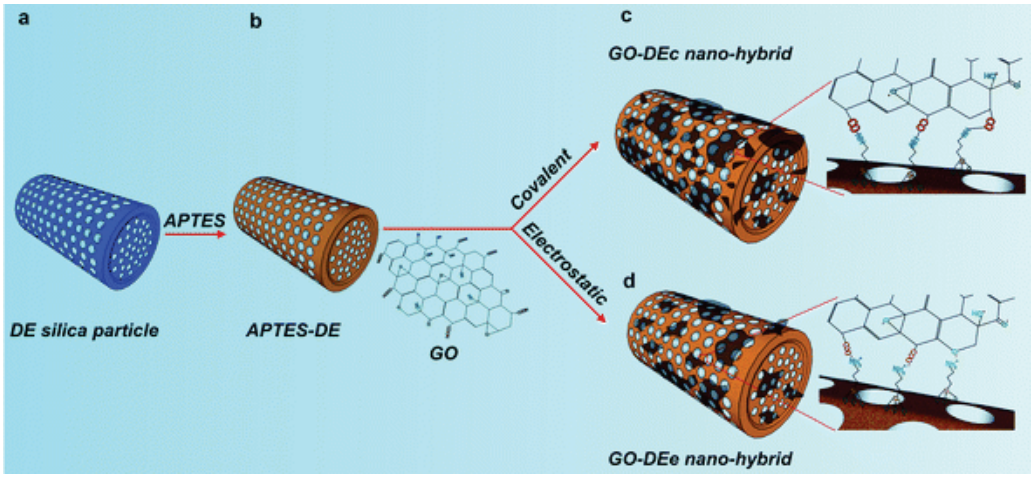

Figure 6. Schematic diagram illustrating the process of fabrication of GO-DE nano-hybrids through electrostatic and covalent attachment routes. a) Plain diatomaceous earth (DE) silica particles, b) organo-silane (APTES) functionalized DE particles, c) GO-DE nano-hybrids fabricated via covalent attachment and d) electrostatic attachment of graphene oxide (GO) sheets. Reproduced from [61] with permission of The Royal Society of Chemistry. Copyright The Royal Society of Chemistry, 2013.

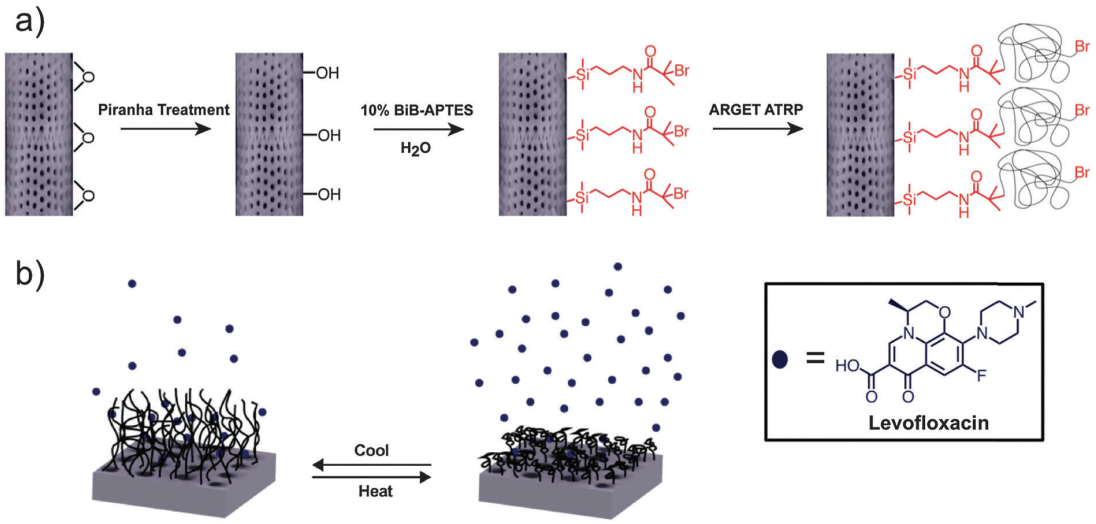

Figure 7. a) Process of diatom biosilica microcapsule functionalisation and b) drug release from thermo-responsive polymer-grafted biosilica frustule. (BiB-APTES: 3-(2-bromoisobutyramido)propyl(triethoxy)silane, ARGET-ATRP: activators regenerated by electron transfer based atom transfer radical polymerisation). Reproduced from [62] with permission of The Royal Society of Chemistry. Copyright The Royal Society of Chemistry, 2015.

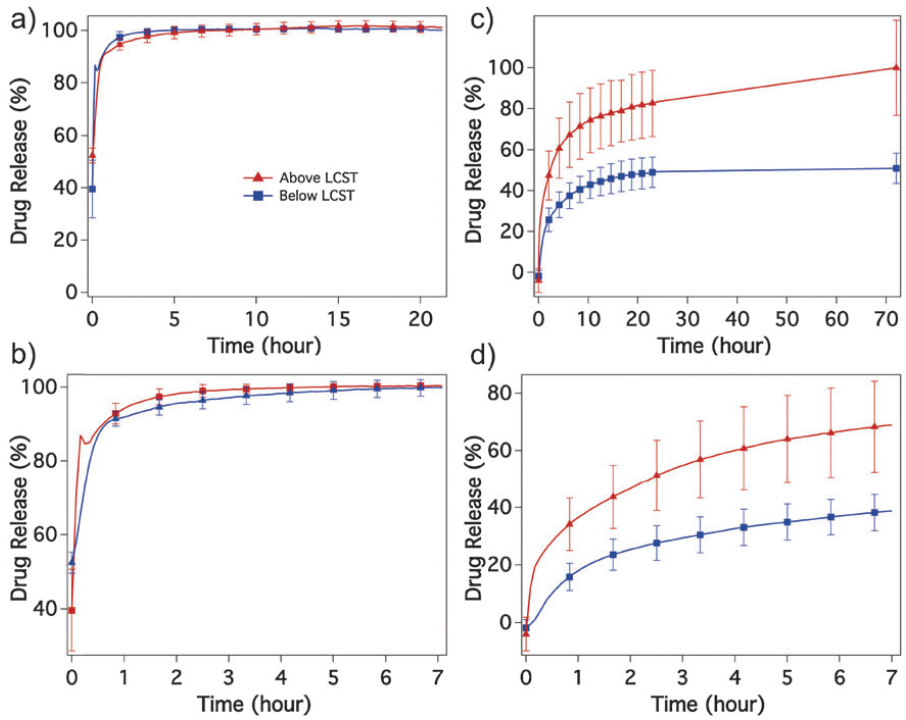

Figure 8. Release curves of levofloxacin from the diatom microcapsules below and above the LCST of the P1 copolymer. a) Total release and b) first 7 hours of release from unmodified microcapsules, (c) total release and (d) first 7 hours of release from P1-modified microcapsules. (P1 mixture of oligo(ethylene glycol)methacrylate copolymers $O(EG)_2MA$ and $O(EG)_4$–5MA in the mole ratio 1/0.16 $O(EG)_2MA/O(EG)_4$–5MA = 1/16). Reproduced from [62] with permission of The Royal Society of Chemistry. Copyright The Royal Society of Chemistry, 2015.

chemical interactions between drug molecules and polymer. Therefore, this approach is compatible with a range of therapeutics, regardless their different chemical structure, where drug release is determined by polymer's characteristics, such as lower critical solution temperature (LCST) in the case of thermo-responsive polymers.

Although the above techniques are successful in modification of native diatom frustules, there is a much simpler way to obtained diatoms with modified physicochemical properties. Growing of diatoms in culture medium enriched in modified agent should cause incurporation of this agent into frustule architecture. Addition of germanium into the culture medium didn't

affect the frustule shape, but it's affected on pore array. It was shown that electroluminescence (EL) devices fabricated from frustule with incorporated germanium exhibit EL emission, while no emission was detected in devices based on native frustule. Frustule with incorporated germanium also has a much higher PL intensity compared to native frustule [63]. Exposure of diatom *C. wailesii* to nickel sulfate ($NiSO_4$) decreases number of pores and increases pore diameter twice as compared to control samples, possibly due to enzyme disruption. A PL spectrum is also changed with significant quenching of PL under exposure to $NiSO_4$ [64]. These results give us hope that it will possible to produced custom tailor devices for desired applications in high yield using cheap processes under mild condition. These results are also important from an ecological point of view. It is known that diatoms are very efficient in photosynthesis. The optical features of their frustule developed during evolution allow them to utilize the whole spectrum of visible light with the same efficiency [14]. As these features can be affected by metals, that are presented in water habitats, which are becoming more and more polluted, diatoms photosynthetic capacity and removal of atmospheric CO_2 can be decreased which may have unforeseeable consequences. Since frustule architecture is sensitive on metals presence in aquatic environment, diatoms may serve as bioindicator of aquatic pollution level.

CONCLUSIONS

Diatoms due their specific porous structure and properties offer great opportunities for numerous bioapplications, such as drug delivery, biomolecules immobilization, bio- and gas sensing. Their great species diversity and species specific highly organized frustule architecture with great possibilities for chemical modifications should enable us to obtained custom tailor devices for specific applications. Due to their easy availability in large quantities and rapid reproduction with requirements of only light and minimal nutrients for growth, diatoms represent cheap source of nanostructured materials. Despite affirmative results in different kinds of applications that are described in this paper, the use of diatoms is still mostly in the field of researches. Although the researches for some kinds of application is at the beginning, the day when diatom based devices will be in widespread use is not far from us. The newest application of diatom frustule as drug carriers gave promising initial results. But these *in vitro* results should be confirmed in both experimental animals and patients. It should be prove the real clinical benefits of using diatom carriers and its biocompatibility. Immobilization of biomolecules should enable development of new delivery systems for biotechnological drugs as well as diatom based biosensors. Low enzyme loading capacity, achieved in the initial studies, is currently bottleneck of this process and should be overcome with further elucidation the mechanisms of diatoms morphogenesis. The main challenge in using of diatoms in bio and gas sensing is to obtain specific and high sensitive sensor capable to distinguish similar substances in complex mixture. This was successful done with attachment of antibodies that have natural ability for high specific binding of target molecules. This opens up possibilities for using diatoms in diagnostic as well as in specific and high sensitive detection of low gases concentration in air pollution monitoring or in different industries.

Acknowledgements

This work was supported by the project No. TR 34007, funded by the Ministry of Education, Science and Technological Development, Republic of Serbia.

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IZVOD

DIJATOMI – MATERIJALI PRIRODNOG POREKLA SA VELIKIM POTENCIJALOM ZA RAZLIČITE BIOAPLIKACIJE

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(Pregledni rad)

Dijatomi predstavljaju jednoćelijske silikatne alge, široko rasprostranjene u vodenim ekosistemima. Osnovnu karakteristiku, po kojoj se izdavajaju ovi organizmi, predstavlja ćelijski zid izgrađen od amorfnog silicijum-dioksida, koji se označava i kao frustula. Visoko organizovana mikro- do nanoporozna struktura, velika specifična površina, prisustvo reaktivnih silanolnih grupa, koje omogućavaju laku hemijsku modifikaciju površine, biokompatibilnost, visoka mehanička otpornost i jedinstvena optička svojstva (fokusiranje svetlosti i luminescencija) čine frustulu dijatoma pogodnim materijalom za razvoj velikog broja različitih sistema, kao što bio- i gasni senzori, sistemi za razdvajanje čestica, sistemi za isporuku gena i lekovitih supstanci, solarne ćelije, baterije i dr. Široka dostupnost dijatomskih silikatnih struktura u vidu fosilnih ostataka dijatoma (dijatomejska zemlja ili dijatomit) uz jednostavnu kultivaciju dijatoma u veštačkim uslovima, čini ove materijale izuzetno ekonomski prihvatljivim. Imobilizacijom različitih enzima u dijatomske frustule, kako primenom genetskog inženjeringa, tako i fizičkohemijskom adsorpcijom, postignuto je povećanje fizičkohemijske stabilnosti, uz očuvanje aktivnosti enzima. Primenom dijatomskih frustula u razvoju sistema za isporuku lekovite supstance, postiže se karakteristično bifazno oslobađanje lekovite supstance, sa početnim brzim oslobađanjem lekovite supstance adsorbovane na površini, koje zatim prati produženo oslobađanje lekovite supstance adsorbovane unutar porozne strukture frustule. Zahvaljujući jedinstvenim fotoluminescentnim svojstvima, koja su podložna promeni u zavisnosti od supstanci prisutnih u okruženju, dijatomske frustule se mogu koristiti za dobijanje optičkih senzora za visoko osetljivu detekciju različitih gasova, ili biomolekula prisutnih u okruženju. Nemogućnost razlikovanja signala koji potiču od molekula slične hemijske strukture uspešno je prevaziđena funkcionalizacijom površine dijatoma različitim antitelima. Različitim hemijskim modifikacijama moguće je prilagođavanje fizičkohemijskih karakteristika dijatomskih frustula za specifičnu namenu. Rezultati do sada sprovedenih istraživanja su pokazali veliki potencijal silikatnih struktura izolovanih iz dijatoma za različite biomedicinske primene, tako da u budućnosti treba očekivati širu komercijalnu primenu ovih materijala.

Ključne reči: Dijatomi • Sistemi za isporuku lekovite supstance • Biosenzori • Gasni senzori • Imobilizacija biomolekula • Ciljana isporuka lekovite supstance