One of the main challenges in modern tissue engineering is to design biocompatible scaffolds with finely tuned porous architecture and capacity to load bioactive molecules that guide the growth and differentiation of the cells during tissue reconstruction. This work proposes a strategy to design porous alginate scaffolds (PAS) with well-tuned architecture by leaching of sacrificial vaterite CaCO₃ microspheres packed in alginate. Pore size and interconnectivity depend on CaCO₃ sphere dimensions and packing as well as alginate concentration. Varying of these parameters, almost hundred percent pore interconnectivity (or, by contrast, a zero pore interconnectivity) can be achieved. Junctions between interconnected pores are about 50–70% of the pore dimensions that provides molecular transport through the PASs potentially ensuring diffusion of nutrition, oxygen and metabolic products when cell seeding. An opportunity to fabricate a multifunctional scaffold is demonstrated by encapsulation of desired macromolecules into the individual pores of a scaffold (is illustrated by dextran loading). Mechanical properties of PASs are found typical for soft and hydrated structures (Young's modulus of 19 ± 15 kPa) which is appropriate for cell seeding. The three cell lines (HeLa, HEK293, and L929) are cultured on different alginate scaffolds to examine cell viability and adhesiveness.

1. Introduction

Polymeric scaffolds are valuable degradable supports offering essential chemical and physical signals to guide growth of biological cells and tissue development.[1] The scaffolds are usually porous to ensure cell infiltration/growth and to avoid diffusion limitations for nutrition and metabolites. Nowadays a large number of techniques to design polymeric scaffolds have been elaborated including gas foaming,[2] microfluidics,[3] solvent/casting particulate leaching,[4] electrosprinning,[5] emulsion freeze drying,[6] 3D bioprinting,[7] etc. These and other studies moved to the forefront of tissue engineering polymer-based and composite[8] gel scaffolds, which can mimic the natural microenvironment of the cells. When designing these scaffolds, the main challenges which are still present are 1) the control over scaffold internal porosity, and 2) loading and distribution of bioactive molecules such as growth factors necessary to stimulate proper cell growth. This is largely dictated by the conditions used in the process of scaffold fabrication: high or low temperature, organic solvents, exposure to gas-liquid or solid-liquid interface, aggressive chemicals.

Alginate hydrogels have been reported for various biological applications[9] and are widely used as polymeric scaffolds that is justified by their biocompatibility and well-defined relation between the hydrogel material properties and biologically related characteristics.[9a,10] These hydrogels can provide not only space and mechanical support for biological cells[10a] but also can be used as reservoirs to supply cells with active molecules, e.g., proteins and growth factors.[11] Loading of the gels

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with biomolecules should be done at mild conditions to ensure biological activity of the encapsulated compound.

The gel is formed due to affinity of alginate molecules to divalent ions (such as calcium) which cooperatively bind between G-blocks of alginate molecules resulting in reversible ionic cross-linking described via “egg-box” model. Not only a solution of calcium ions but also solid CaCO$_3$ spheres can be used as a source of ions for alginate gelation. The latest has been utilized for the fabrication of alginate scaffolds mostly controlling the dissolution of the CaCO$_3$ by self-hydrolyzing polymer (D-glucono-$\delta$-lactone) used as an additive. When no additives used, harsh acidic conditions are generally required. At the same time, (bio)molecule encapsulation by means of templating using decomposable CaCO$_3$ microspheres is nowadays a popular approach to particulate fragile bioactive molecules because of mild decomposition conditions for the carbonate spheres (pH below 6 or chelating agents such as EDTA). In addition, CaCO$_3$ microspheres can be easy prepared with a controlled diameter in micrometer or even sub-micrometer range. Anhydrous calcium carbonate can exist in three polymorphic forms (calcite, aragonite and vaterite). Among these forms, the vaterite attracts especial attention, because its highly developed mesoporous internal structure with an average pore size of tens of nanometers allows one to load molecules of interest during the CaCO$_3$ microsphere synthesis (co-precipitation) or by loading into the pre-formed spheres.

Recently demonstrated approaches have shown the opportunity to utilize the CaCO$_3$ microspheres for the tissue-like cell assembly, improving alginate printability for biofabrication via its pre-cross-linking, and fabrication of alginate gels with micrometer-sized pores. In this work, we have used the templating on vaterite microspheres to fabricate 3D porous alginate scaffolds (PAS) with controlled architecture and localization of the molecules within the scaffold. In contrast to previous reports, one-step fabrication procedure based on the leaching of the CaCO$_3$ at mild conditions (pH 7.4) and without the use of hydrolyzing additives is proposed. By employing optical and atomic force microscopies as well as colloidal probe we focus on gel internal structure (porosity) and mechanical properties, which are indispensable for cell proliferation and growth in the porous alginate gels to be used as scaffolds for cell studies. Culturing of the three cell lines on different alginate scaffolds is performed in order to examine their viability and adhesiveness. Encapsulation of model biomolecules (dextran) into the pores of scaffold by pre-loading into the CaCO$_3$ microspheres is addressed.

2. Results and Discussion

2.1. Design of PAS

Figure 1 presents schematics of the developed approach to fabricate the PAS. At first, CaCO$_3$ microspheres were dispersed in highly concentrated alginate solution and packed (if required) by means of one of the approaches described in the experimental section. The following dissolution of the carbonate microspheres was resulted in the formation of the porous hydrogel. Structure of the scaffold including pore interconnectivity is further considered.

2.1.1. Pore Hollowness

To examine if the pores formed in the scaffold are hollow, the prepared PASs were stained with Rho 6G and analyzed by CLSM (Figure 2). As evident from the fluorescence profile taken across the scaffold (Figure 2), the fluorescence signals coming from the pore’s interior and from the outside of the PAS are the same (the small difference can be caused by Z-direction limitations in optical resolution of the microscope) meaning that the pores are hollow. The obtained SEM images of the
could not penetrate into the nanopores of CaCO₃ microspheres G-blocks of alginate molecules forming large aggregates, which solution being mixed with. The released Ca²⁺ ions from the surface of CaCO₃ spheres into the surrounding solution being mixed with. The released Ca²⁺ ions cross-link G-blocks of alginate molecules forming large aggregates, which could not penetrate into the nanopores of CaCO₃ microspheres (tens of nm). Thus, gelation process took place only outside the spheres providing formation of PAS with hollow pores formed on the places of dissolved vaterite microspheres.

![Figure 2. Fluorescence and optical transmittance images of the middle part of PAS formed using 33-μm-CaCO₃ microspheres packed via centrifugation (above) and of nonporous alginate scaffold (nonPAS) (below). Prepared scaffolds were stained with Rho 6G. Fluorescence profile corresponds to the solid white line. The arrows highlight different fluorescence intensities between the pores of the scaffold indicating different size of junction between them.](image)

The hollowness of the pores can be explained by the following reason. When being dispersed in pure water, alginate molecules possess the hydrodynamic diameter of about ten nm (as measured by DLS). However, when mixing of CaCO₃ spheres with alginate solution, polymer molecules always tend to aggregate forming assemblies with the diameter of about 1 μm. This fact can be explained by the partial release of Ca²⁺ ions from the surface of CaCO₃ spheres into the surrounding solution being mixed with. The released Ca²⁺ ions cross-link G-blocks of alginate molecules forming large aggregates, which could not penetrate into the nanopores of CaCO₃ microspheres (tens of nm). Thus, gelation process took place only outside the spheres providing formation of PAS with hollow pores formed on the places of dissolved vaterite microspheres. lyophilized PAS have also demonstrated the porous gel matrix with the highly interconnected structure.

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The same was observed for smaller CaCO₃ spheres (diameter of 11 ± 1 μm): the average pore size in this case was 10 ± 1 μm. Interesting, when using higher alginate concentration (5%), an average diameter of the pores (6.8 μm) was found much below the size of CaCO₃ vaterite when using 11 μm spheres (Figure S1, Supporting Information). Such reduction in pore size can be caused by the external osmotic pressure generated by calcium ions (and water molecules coming with the ions) during cross-linking of alginate molecules. This osmotic pressure can lead to the gel swelling compressing the pores (Figure S2, Supporting Information). If consider rather thin gel (films of 20–50 μm in height) the crosslinking goes fast, and the formed gel swells without any limitations keeping a pore diameter exactly equal to a size of CaCO₃ microspheres. However, if gelation takes place in an eppendorf tube of a restricted volume, the osmotic pressure generated by the swelling gel network may affect the internal structure via shrinking of the pores (detailed description is given in the caption to Figure S2, Supporting Information). For these scaffolds with the shrinked pores (Figure S1a, Supporting Information), the lower content of interconnected pores was found (38%, Table 1) that can be also explained by the pore shrinkage supporting the assumption regarding an effect of the osmotic pressure. Pore interconnectivity is considered in the next section. It is of note that lower or higher alginate concentrations – namely 1% and 10% w/v – were not applied because in these cases either no stable pores were formed or alginate solution was too viscous for good dispersion of CaCO₃ microspheres, respectively.

Size and packing of CaCO₃ microspheres affected not only the size of the formed pores but also carbonate elimination time. The smaller the spheres were, the longer their dissolution occurred. Ceteris paribus, 11-μm spheres were fully dissolved.

### Table 1. Characteristics of PAS formed using 11 and 33 μm CaCO₃ microspheres dispersed in 3–5% alginate solution and packed by “centrifugation” approach.

| CaCO₃ size, μm | Alginate concentration, % | Content of interconnected pores, % | Diameter of interconnected pores, μm | Connection length, μm | Ratio connection length/pore diameter |
|----------------|---------------------------|------------------------------------|---------------------------------------|-----------------------|-------------------------------------|
| 11 ± 1         | 3                         | 52                                 | 10.1 ± 1.1                            | 7.1 ± 0.9             | 0.70 ± 0.10                         |
| 11 ± 1         | 5                         | 38                                 | 6.8 ± 1.0                             | 5.1 ± 0.5             | 0.75 ± 0.07                         |
| 33 ± 9         | 3                         | 57                                 | 30.8 ± 5.5                            | 18.0 ± 4.6            | 0.58 ± 0.50                         |
| 33 ± 9         | 5                         | 55                                 | 30.4 ± 6.5                            | 16.7 ± 4.7            | 0.55 ± 0.56                         |

*The content of interconnected pores (in %) presented in the table is calculated only from the CLSM images (scans) in XY-plane but not in Z-plane due to lower resolution in that direction. The real number of interconnected pores is much higher as evident from the free diffusion of high-MW macromolecules between the micropores (Figure S4, Supporting Information).
after 36 h while the 33-µm ones were removed after 22 h. Most probably, the longer dissolution time is required for smaller spheres due to their denser packing and more diffusion limitations (for acid or buffer) to diffuse through the smaller spaces between the 11-µm spheres.

2.1.3. Pore Connectivity

Packing of CaCO₃ microspheres performed in this work (see Experimental Section for details) influenced porosity of the forming scaffolds. When CaCO₃ spheres were packed in alginate solution (inducing multiple connections between the spheres), the forming after microsphere’s removal pores were also found to be connected with each other (Figure 3a). Important, without packing of CaCO₃ spheres, the pores in the fabricated scaffold were free-standing (Figure 3b). Open junctions between the pores are of high importance for cell culture. Indeed, the scaffold should possess interconnected structure to provide a good diffusion of nutrition and metabolite products toward/outward the growing cells. In this section, we focus on the pore interconnection analysis.

As evident from the fluorescence profile shown in Figure 2, a distance between the hollow pores varies giving either low or high fluorescence signal (indicated with arrows in places of the pore connections). However, it is hard to conclude about the pore interconnectivity from images only because a resolution of CLSM in Z-plane is lower compared to XY-plane. Even if there is no visible junction between two neighbor pores, the real depth of this junction can be larger as concluded from the fluorescence profiles. Therefore, the analysis of pore interconnectivity requires an introduction of a certain approach to distinguish between interconnected and free-standing pores. To analyze the experimental data, the following model was proposed (Figure 4 and Figure S2: Supporting Information). Two neighbor pores are considered as free-standing if the fluorescence intensity h in the place of connection is higher than a half of the fluorescence level H of the surrounded hydrogel (h > H/2). In all other cases (h ≤ H/2) two pores are considered as interconnected (having an open junction). For free-standing

![Figure 3](image-url)

Figure 3. SEM images of two lyophilized PASs: a) the scaffold with highly interconnected pores with the average pore diameter of 31 ± 7 µm; b) the scaffold with free-standing pores with the average pore diameter of 10 ± 1 µm. Insets present SEM images of the CaCO₃ microspheres with diameters of 33 ± 9 and 11 ± 1 µm used for fabrication of the scaffolds shown in (a) and (b), respectively.

![Figure 4](image-url)

Figure 4. a) Schematics of interconnected pores demonstrating how to distinguish between free-standing and interconnected pores. b,c): Lateral and longitudinal profiles taken across the pores according to the corresponding lines. (b): Pores are considered as free-standing if the fluorescence signal in the place of pore connection h is higher than a half of a maximum fluorescence H in the surrounded PAS (h > H/2). Pores are interconnected if fluorescence in the place of pore connection h ≤ H/2. c): The junction dimensions x has been estimated as the full width at half maximum.
pores the fluorescence signal in the place of pore connection is by about 25% lower than that from the hydrogel, while for interconnected pores the fluorescence in the junction point drops down by about 75% (see example images and corresponding profiles in Figure S2, Supporting Information). Due to such a dramatic difference between the depths, one may state that the pores identified here as interconnected have definitely an open junction.

As calculated used the described model, the number of interconnected pores in PASs was found to be depending on the packing approach applied to CaCO₃ microspheres (Table 1). Rather low number of interconnected pores (about 9% and 14%) has been found for “sedimentation” and “concentration” packing approaches, respectively. However, a number of interconnected pores was up to hundred percent when centrifugation of microspheres. Indeed, the additional experiment performed to examine diffusion of macromolecules between the pores of PASs demonstrated an instant penetration of dextranFITC (MW 2000 kDa) macromolecules into the pores over the whole scaffold (Figure S4, Supporting Information). Most probably, an additional force applied by strong centrifugation allows to pack the microspheres compact and in a tight contact with each other forming PASs with highly interconnected structure. One has to note that 2% of the microspheres used to prepare the gel were initially coupled to each other (as counted from the optical transmittance images by analysis of thousand microspheres). Most probably, this took place during the co-precipitation when two microspheres growing in vicinity to each other may merge to form a couple. Such couples will also form interconnected pores. However, a number of such couples is much lower compared to the high percent of interconnections found in PASs meaning that these couples do not play a role in pore interconnectivity.

At the same time, it is hard to quantify dimensions of the open junction between pores using CLSM even if use a very precise scanning step (due to rather low resolution in Z-plane as discussed above). The junction dimensions x can be only approximately determined when taking a profile along the place of pore connection as shown in Figure 4 and Figure S5 (Supporting Information). The average size of junction between interconnected pores was found to be at least a half of the pore diameter (Table 1 and Figure S6: Supporting Information). One could expect that the enlargement of the space between the connected pores took place during gelation due to the additional forces (Figures S7 and S8, Supporting Information). Most probably, the gel pressure brought the connected pores together which leads to pore fusion increasing the junction length (Figure S7b, Supporting Information). To the contrary, if the pores were not connected, the osmotic pressure could bring them apart because the pressure will be generated between these pores as well (Figure S7a, Supporting Information). As an alternative to the mechanism based on pore fusion, mechanical destruction of the junction between the pores by releasing ions and molecules may be expected (Figure S7c, Supporting Information). In this case, generated pressure may mechanically destroy rather thin parts of the gel boundaries between the formed pores. However, the probability of this mechanism seems to be much lower (details are given in the caption to Figures S7 and S8, Supporting Information).

2.1.4. Localization of DextranFITC in the Scaffold

For bioapplications, scaffold should not only possess a well-determined structure providing a space for cell proliferation, but also should allow encapsulation of desired macromolecules such as proteins and growth factors needed for cell growth. In the previous sections, a precise control over the scaffold porosity has been demonstrated. Now the possibility of the localization of the molecules of interest in the scaffold will be shown.

To fabricate a multifunctional PAS possessing both a controlled porosity and loaded with macromolecules, two types of CaCO₃ spheres were used: i) empty 20 ± 5 µm vaterite and ii) dextranFITC-loaded 3 ± 1 µm vaterite. In is of note that fluorescently labelled dextran has been chosen for the visualization of molecule distribution within the scaffold at microscopic level. The empty and loaded microspheres were mixed at a ratio of 3:1 and packed in 5% alginate solution applying centrifugation. The following TRIS-mediated sphere dissolution (see experimental section) resulted in the formation of PAS with locally encapsulated macromolecules (Figure 5). Notably, dextranFITC remains entrapped inside the pores formed after the elimination of 3 ± 1 µm vaterite crystals and does not leak out of these pores allowing its controlled localization. This demonstrates how easy the required compounds can be loaded into the scaffold via pre-encapsulation into CaCO₃ sacrificial spheres. Important, one can prepare different butches of CaCO₃ spheres loaded with various compounds to be encapsulated in a scaffold. In this way, a scaffold will contain several types of drugs distinguished between different pores.

2.2. Mechanical Properties of PAS

Due to the importance of mechanical characteristics of a scaffold for cell adhesion, colloidal probe was applied to PAS. Figure S9 (Supporting Information) demonstrates mapping of the same position on the scaffold for its height and elasticity modulus. As evident from the images and from the profiles taken, there is no correlation between morphological features of the gel and its softness. The “hill” in the right part in Figure S9 (Supporting Information) corresponds most probably to a pore formed after elimination of 11 µm CaCO₃ microsphere (according to dimensions of the “hill”). Perhaps, colloidal probe technique probed just an upper part of the gel that does not depend on the PAS morphology. Young’s modulus varies in the range from 10 to 40 kPa giving an average value of 19 ± 15 kPa. This value of softness is typical for soft and hydrated structures[100,21] and is on the border of spreading of the model cells such as fibroblasts on soft polyelectrolyte multilayers. These findings are of importance for the following cell seeding on PAS.

2.3. Cell Culture

As described in the experimental section, three types of the cells (HeLa, HEK293, and L929) were seeded onto different types of alginate scaffolds in order to examine cell viability, adhesivity and proliferation. These are adherent cells that
represent two most commonly used cell lines, epithelial HEK293 and HeLa, and the fibroblasts L929 cells derived from the adipose tissue. The performed control experiment (cell seeding without scaffolds on the non-modified surfaces of the chamber, Figure S10: Supporting Information) revealed good cell proliferation demonstrating the chosen conditions to be appropriate for cell growth. However, the cells seeded onto the surfaces of different scaffolds demonstrated various behavior (Figure 6 and Figure S11: Supporting Information).

At first, all the types of cells were not adhesive to the scaffolds fabricated at acidic conditions (Figure 6 and Figure S11: Supporting Information) notwithstanding the long-term incubation of these scaffolds in CaCl2 solution (performed to exchange the possibly cross-linking alginate H+ ions to Ca2+ ions) and then in PBS. After 3 days of incubation, only single round-shaped cells were found on surfaces of the scaffolds (Figure S12, Supporting Information) and no improvement was found after 7 days. Thus, the conditions of scaffold’s fabrication are of importance for the following cell attachment/proliferation (not only for the pre-encapsulated bioactive compounds into the scaffold). Most probably, the performed post-treatment of the scaffolds in CaCl2 and PBS did not provide the completed removal of acid making scaffolds irresponsibly unfavorable for cell adhesion.

Interesting that all the types of cells demonstrated poor adhesion to the alginate scaffolds formed at pH 7.4 but without pores (nonporous alginate scaffolds, or nonPAS, Figure S13: Supporting Information). Only a small amount of single round-shaped cells was found (mostly attached to some defects of nonPASs such as scratches formed during the fabrication process) (Figure S14, Supporting Information). At the same time, a few islands of cells were found (as shown in Figure S14 (Supporting Information) for L929 cells). However, these areas contained a large number of dead cells. Moreover, alive cells did not continue to proliferate on the surface of the scaffolds until the next visualization made on the 7th day. This indicates poor conditions for cell proliferation.

At last, after 1 week the cells formed a monolayer almost everywhere on the surfaces of PASs fabricated in buffer solutions (Figure 6 and Figure 7). The treatment of scaffold with PLL TRITC before cell seeding (performed to change a charge of a scaffold and, probably, enhance cell adhesion) did not significantly change the cell growth; the adhesion of cells to the surface was only slightly improved (higher amount of attached cells on the 3rd day). Independent on the treatment with PLL, on the 7th day the cells formed a monolayer on the available scaffold’s surfaces (Figure 7a,b). In some areas of PASs and also on the scaffold boundaries (Figure S15, Supporting Information) cell overgrowth and partial death were observed. The percentage of dead cells proliferated on the scaffold surface was found to be 19% at highest. At the same time, the amount of dead cells raised up to 48% when poor conditions for cell culture such as cell overgrowth (Figure S15, Supporting Information).

It is of note, CLSM images of HEK293 cells on PASs (produced at pH 7.4) are not provided here, because PASs were almost fully decomposed after incubation (Figure S11, Supporting Information). Indeed, the observed changes in color of the media (where HEK293 cells with the scaffolds were incubated) evident that the cell division was going intensively as well as for the other two cell lines. However, after incubation with HEK293 the scaffolds were so destroyed, that they were not incapable to be replaced and stained for further analysis by CLSM. Thus, at the examined conditions HEK923 cells caused decomposition of PASs after 1 week. For the other two cell lines (HeLa, L929) decomposition time was longer being at least

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Figure 5. a) Light transmittance, b–d) fluorescence CLSM images of the internal structure of PAS (stained with Rho 6G, red fluorescence in b,d) with encapsulated dextran FITC (MW 70 kDa, green fluorescence in c,d). Scaffold was formed in TRIS buffer using two types of CaCO3 microspheres mixed and centrifuged in 5% alginate solution: i) empty 20 ± 5 µm vaterite and ii) dextranFITC-loaded 3 ± 1 µm vaterite.

Figure 6. Diagrams showing the density of alive a) HeLa and b) L929 cells on different types of alginate scaffolds (with identical composition and dimensions) after 7 days: PASs formed in HCl (acidic pH), PASs fabricated in buffer (pH 7.4) and modified/not modified with PLL; nonPASs formed in CaCl2 and pre-modified/not modified with PLL.
2 weeks. This means that after that time PAS will be self-decomposed leading to release of all the therapeutics pre-encapsulated into the pores of a scaffold. This long-term scaffold dissolution would be a benefit feature when application of a scaffold in vivo (for example, as a wound dressing or surgical barrier). This issue is a topic of our next study.

3. Conclusions

In this work, PASs with fine-tuned internal structure and with capacity to load the molecules of interest were designed by leaching of the CaCO3 microspheres packed in alginate solution. Dimensions and interconnectivity of the pores were pre-determined by size and packing of sacrificial CaCO3 microspheres. The average pore dimensions (31 ± 7 and 11 ± 1 µm) were found to be identical to those of the used sacrificial microspheres (33 ± 9 and 10 ± 1 µm, respectively). Maximal pore interconnectivity of about hundred percent (as evident from diffusion of macromolecules between the micropores) was achieved when packing of the microspheres by centrifugation. The average size of open junction between the pores was found to be 50–70% of the pore size (that is 5–7 and 16–18 µm for 11 and 33-µm-CaCO3 microspheres, respectively). Such large junctions may be explained by fusion of connected pores (due to high osmotic pressure generated in the gel upon microsphere dissolution). High pore interconnectivity should provide molecular transport through PAS potentially ensuring diffusion of nutrition, oxygen and metabolic products when cell seeding.

We demonstrated the opportunity of the entire fabrication of PASs in a buffer solution (pH 7.4). Up to the best of our knowledge, this is the first example of implementation of CaCO3 leaching technique at mild conditions (neutral pH) without a use of hydrolyzing agent that was shown of high importance when culturing of cells. Indeed, the seeding of HeLa, HEK293 and L929 cell lines revealed the importance of both scaffold’s porosity and conditions used to fabricate a scaffold. The cells demonstrated good adhesivity and proliferation when seeding on PASs fabricated in a buffer at pH 7.4. At the same time, the cells were not able to attach the surface and grow on PASs fabricated at acidic conditions, and were poorly adhesive to flat surfaces of scaffolds prepared without the pores.

An opportunity to fabricate a multifunctional PAS was demonstrated by encapsulation of dextran-FITC macromolecules (MW 70 kDa) into the individual pores of a scaffold. Loading of the pores of a scaffold was performed using the advantage of mesoporous structure of the vaterite, which allowed pre-encapsulation of macromolecules into sacrificial calcium carbonate, so that the desired amount of drug can be loaded. Moreover, various therapeutics can be simultaneously encapsulated into different pores of a scaffold. Importantly, both scaffold fabrication and loading of macromolecules were performed at mild conditions that is essential when dealing with pH-sensitive biomolecules.

4. Experimental Section

**Materials:** The used chemicals are: alginic acid sodium salt from brown algae (MW 12–80 kDa, viscosity 4–12 cP for 1% H2O solution, Sigma-Aldrich), calcium carbonate (CaCO3) microparticles, hydrochloric acid (HCl, Merck Chemicals), Rhodamine 6G (Rho 6G, Sigma-Aldrich), dextran-FITC (MW 70 and 2000 kDa, Sigma-Aldrich), TRIZMA base (TRIS, Sigma-Aldrich), HEPES buffer (HEPES, ROTH), phosphate buffer saline tablets (PBS, Sigma-Aldrich). All chemicals were used as purchased without further purification; all solutions were prepared using Millipore water (18.2 MΩ cm).

**Fabrication of CaCO3 Microspheres:** Two types of CaCO3 vaterite microspheres were synthesized using a co-precipitation previously described in the works.[15a,16b] In general the size of microspheres can be varied during precipitation by changing the preparation conditions (e.g., concentrations of the salts used, saturation rate and time, temperature of solutions during the reaction, etc.).[15a] In this work, temperature of the solutions used was varied in order to obtain different dimensions of microspheres (all the other parameters were kept constant). To obtain smaller spheres, all the solutions were used and all the reactions were performed at room temperature. To prepare larger vaterite spheres,
all the solutions were cooled down to the temperature being 4 °C; the synthesis was also carried out in a cool room at temperature 4 °C. To prepare vaterite spheres, a glass beaker with 9 mL of Millipore water was placed on the magnetic stirrer. Under constant stirring, the equal volumes of Na₂CO₃ and CaCl₂ salt solutions (1 m, 3 mL of each) were simultaneously added to the water inducing nucleation of CaCO₃. After 30 s a stirring was stopped, and the suspension was kept for a time being 5 and 45 min (for smaller and larger vaterite spheres, respectively) to complete growth of the CaCO₃ nuclei. The fabricated CaCO₃ microspheres were rinsed with Millipore water to remove the unreacted constituents. After all, CaCO₃ microspheres were dried at 90 °C over 1 h and stored at room temperature. Size distributions of the fabricated spheres were 10–12 and 15–50 µm (as calculated from SEM images of microspheres), and the average diameters of the synthesized microspheres were 11 ± 1 and 33 ± 9 µm, respectively. These samples are mentioned in the text as CaCO₃ microspheres of 11 and 33 µm, respectively.

**Loading of CaCO₃ Microspheres:** In addition to the two above-described types of CaCO₃ vaterite, small microspheres loaded with dextranFITC were synthesized in order to demonstrate the possibility to encapsulate macromolecules into the individual pores of a scaffold. To prepare small dextranFITC-loaded CaCO₃ spheres, a glass beaker (a total volume of 10 mL) was filled with 2.5 mL of dextranFITC solution in TRIS (1 mg mL⁻¹). The beaker was placed on the magnetic stirrer and a constant stirring was induced. The equal volumes of Na₂CO₃ and CaCl₂ salt solutions (1 mL, 0.625 mL of each) were simultaneously added to the beaker inducing nucleation of CaCO₃. After 10 s a stirring was stopped, and the suspension was kept for 5 min to complete the growth of CaCO₃ spheres. Fabricated microspheres were sedimented, the supernatant was removed and spheres were rinsed with Millipore water to remove the unreacted constituents. After all, CaCO₃ microspheres were dried at 90 °C over 1 h and stored at room temperature in black nontransparent dye water solutions (0.1–1 mg mL⁻¹) containing 15·10⁻³ m NaCl or with 0.1 m HEPES, pH 7.4. Buffer solution was stirred constantly inducing the flow accelerating ion transport and dissolving CaCO₃ spheres. When all the spheres were dissolved (usually after 40–48 h, gel became transparent), the fabricated scaffold was replaced in PBS containing 0.05 m CaCl₂ (to avoid spontaneous dissolution of the scaffold with time) and further used for cell experiments.

**Colloidal Probe Measurements:** Colloidal probe measurements were done using Nanowizard I AFM instrument (JPK Instruments AG, Berlin, Germany). Samples were measured in Petri dishes filled with 1 mL HCl. Uncoated silicon cantilevers (CSC 12, Micromesh, Estonia) with nominal spring constant of 0.2 N m⁻¹ were used and silica probes of 20 µm in diameter glued to the apex of the cantilevers. Each scan was done with 16×16 data points. The resulting force curves were evaluated using the Hertz – Sneddon force-deformation theory.

**Dynamic Light Scattering:** DLS measurements of hydrodynamic diameter of alginate molecules were done using Zetasizer Nano ZS (Malvern Instruments Limited, Worcestershire, UK). Size measurements were performed at 25°C using Disposable cuvettes UV-Cuvette Micro
759 200 of 70 µL-volume (Brand GMBH, Germany). Hydrodynamic diameter was determined for the alginate molecules dispersed in Millipore water at concentration of 0.1%, w/v. A set of three measurements was performed to determine the average hydrodynamic diameter. During DLS measurements of hydrodynamic diameter, the polydispersity index was estimated.

Cell Seeding and Cultivation: Three types of cells, namely HeLa (human, epithelioid cervix carcinoma), HEK293 (human, primary embryonal kidney), and L929 (mouse fibroblasts) were chosen to be cultured onto the PASs in order to examine the cell viability and proliferation. The following alginate scaffolds were used for cell culture:

i) To examine the effect of the approach (used for scaffold fabrication) on cell proliferation, cells were seeded on PASs produced in TRIS buffer and onto PASs with the same structure and composition but prepared in HCl.

ii) To examine an effect of porosity of the scaffold on cell proliferation, cells were seeded on alginate scaffolds with the same composition and dimensions but without pores. Nonporous alginate scaffolds (nonPAS) were prepared by merely addition of CaCl2 solution onto the thin films of alginate solution (shaped into the stripes using the same hand-made substrate shown in Figure S16, Supporting Information).

iii) Charge of the scaffold (alginate is always negatively charged) can also influence the cell proliferation. In order to examine the effect of scaffold’s charge on cell proliferation, part of the PASs prepared in TRIS buffer were incubated for 1 h in a solution of PLLTRITC (MW 15–30 kDa) before cell seeding. This incubation resulted in the uniform distribution of the PLL molecules over the whole volume of scaffolds.

Cells were cultivated on the above-described scaffolds in Dulbecco’s Modified Eagles Medium (DMEM, Sigma, Germany) supplemented with 10% (for HeLa and L929) or 15% (for HEK293) v/v fetal calf serum (FCS), 2% L-glutamine, and 1% v/v Penicillin Streptomycin. For these experiments, ibidi chambers (a µ-Slide suitable for co-cultivation assays in combination with microscopy, Cat. No. 81 801) were used. To prevent the cell growth outside scaffolds, the surface of ibidi chambers was previously treated with 10% v/v Pluronic F (modified by Pluronic F-68, Sigma). For this purpose, the chamber filled with 10% solution of Pluronic in H2O (filtered by syringe filter with 0.22-µm pore size) was incubated at 37 °C over 1–2 h followed by sterile PBS. The example of ibidi chamber with different PASs is shown in Figure S17 (Supporting Information). The prepared scaffolds were rinsed with sterile PBS, cut into equal pieces with different PASs is shown in Figure S17 (Supporting Information). The cells were stained with calcein and propidium iodide prior CLSM analysis.

The medium was changed daily within the first 3 days and then every 2–3 days. The cell adhesion and proliferation were examined using CLSM on the 3rd, 7th, and 14th day of cultivation. To distinguish living and dead cells, they were stained with calcein and propidium iodide prior CLSM analysis.

To check if the chosen conditions are appropriate for cell culture, the cells were seeded by the same algorithm in the minor wells of non-modified chambers (no scaffolds). Cell growth and adhesivity was monitored on the 3rd day by CLSM.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

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

Keywords

CaCO3, cell culture, encapsulation, hydrogel, porosity

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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