Influence of Pore Space Hierarchy on the Efficiency of an Acetylcholinesterase-Based Support for Biosensorics

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The influence of a hierarchically structured pore system of a silica sol–gel support for application as a functional component in an acetylcholinesterase-based enzyme array, with respect to its efficiency (response time, in particular) is investigated. Careful adjustment of synthesis parameters and a novel drying method allow to prepare monolithic silica sol–gel membranes with monomodal or hierarchical pore structures. These supports enable direct comparison regarding the influence of morphological properties on maximum acetylcholinesterase (AChE) loading by a membrane and on the apparent reaction rate of the AChE-catalyzed degradation of acetylcholine at identical enzyme loading. It is shown for the first time that the hierarchical, meso-macroporous material is superior over the monomodal structures (of either mesopores or macropores) regarding combined functionality and transport efficiency, as reflected in the apparent reaction rates. The advantage of the mesopores in a hierarchical system is manifested in higher maximum enzyme loading than for purely macroporous material, while the presence of macropores results in less obstructed transport that for a purely mesoporous material, which in turn reduces the response time.

1. Introduction

Due to their potential for miniaturized designs, biosensors are a cost-effective alternative to classical analytical platforms.[1] The spatial proximity of biological (functional) components, transducer, and signal converter, as well as the integration of a reaction unit optimized to handle small sample amounts are advantageous over conventional detection and characterization schemes. Biosensors are not only used in clinical diagnostics but also in environmental analysis, military technology, food science, and process control.[1–6]

The enzyme class of cholinesterases occurs in all multicellular organisms and is one of the most important enzyme classes in biosensorics next to the gluco- and galactosidases. Acetylcholinesterase (AChE), the best-known representative of the cholinesterases, acts in the vegetative and central nervous system and in neuromuscular synapses in the transmission of stimuli. To guarantee the transmission of stimuli to the neurons in a short time, the degradation of acetylcholine (ACh) into choline and acetic acid belongs to the fastest reactions. Because of this vital effect of AChE also in the human body, it is important that the mechanism of stimulus transmission is not disturbed.[6–7] The degeneration of the nervous system can cause diseases like Alzheimer’s and forms muscle weakness, which can as well be attributed to a disturbed AChE mechanism.[8] Some pesticides and various chemical warfare agents also rely on the inhibition of the AChE mechanism, so that environmental influences (contaminated food or drinking water) also contribute to enzyme inhibition and may even lead to paralysis of the muscles and death of the organism.[9] With an AChE-based sensor, decisions can be reached about the effect of a medical product, of pesticides, or chemical warfare agents on humans and animals. Therefore, AChE-based sensors are used at early stages of drug and plant protection agent development, as well as in environmental and food analysis for the detection of pesticide residues in food or drinking water samples.[10–12]

Proper functioning of AChE-based sensors relies on a constant enzyme activity, as seen in the substrate decomposition under defined reaction conditions. The degradation of ACh takes place by ester hydrolysis into choline and acetic acid. AChE acts as catalyst and is immobilized on a support for better storage and transport, easier sensor handling, and straightforward separation from analytes.[11,14] The enzyme-support assembly is directly connected to a transducer, for example, an optoelectronic sensor or potentiometric electrodes.[35]

To guarantee optimal biosensor function, high demands are placed on a potential support for immobilization of the biologically active component. AChE is an ellipsoidal molecule of about 4.5 nm × 6.0 nm × 6.5 nm. The β-sheets are arranged in slightly convex form and surrounded by 14 α-helices similar to a sandwich structure. This arrangement of the secondary
structure enables formation of a slightly twisted, helical tertiary structure of the molecule, which requires a pore size of the support of at least 10 nm so that unfolding of the enzyme and thus its activity are not impaired.[37] In addition, the support material should have a high specific surface area (for high loading capacities) combined with high mechanical and thermal stability. Due to these requirements, porous materials are often the first choice. In addition to agarose and polymers such as acrylamide or polyethylene glycol, nanoparticles, or nanoporous layers of gold, titanium dioxide, zirconium, or cadmium sulfide and silicon dioxide are often used in biosensors.[15,18–20] In this case, additional demands are placed on the support regarding little obstructed transport of solutes within the pore system, because short response times should remain one of the main advantages of biosensors.

The combination of mesopores (with large specific surface area for reaction) and macropores (highly permeable flow-through pores enabling advection-dominated transport) as hierarchical architecture in a support material ensures optimized mass transfer in many applications.[21–24] Hierarchical supports can be prepared, for example, via thermally-induced phase separation in sodium borosilicate glasses. Hierarchy is also realized by employing sintering processes in combination with the replica process using tube bundles or with foaming processes.[25–27] Hierarchical silica materials can be prepared based on sol–gel chemistry, which is achieved by polymer-induced phase separation during the sol–gel process.[28–32] Sol–gel materials are already employed in several applications including biosensors and microfluidic devices, but often with monomodal pore structure. In microfluidic chips for DNA isolation or as flow-through cells for an optical biosensor and also in combination with AChE the devices are operated using purely monomodal (mesoporous) sol–gel materials.[33–36] We therefore study closer to the performance in such applications. In this work, we investigate the impact of a hierarchical pore system on the efficiency (mass transfer resistance and response time) of a functional support based on AChE for biosensors compared to materials with monomodal pore systems. By adapting a general sol–gel synthesis protocol purely mesoporous, purely macroporous, and bimodal (meso-macroporous) supports have been synthesized. These three basic pore architectures were compared in a model system to determine the maximum loading of AChE as well as the apparent reaction rate of the AChE-catalyzed degradation of ACh at identical enzyme loading. We highlight the conditions when a hierarchical support is indispensable and when a purely macroporous material suffices.

2. Results and Discussion

2.1. Synthesis of Porous Silica Membranes

To identify positive effects of a hierarchical pore structure compared to monomodal pore systems, we adapted the strategy from Figure 1. A hierarchical (meso-macroporous) support is required when higher enzyme loadings are needed than realizable with a purely macroporous material. They can be achieved by an increase of the specific surface area with the additional mesopores inside the silica skeleton.

Starting sols comprised sulfuric acid solution with tetraethyl orthosilicate (TEOS) as silica precursor, water as solvent, and, for the macroporous and hierarchical materials, poly(ethylene oxide) (PEO) as porogen.[28] After several synthesis steps (gelation, hydrothermal treatment, washing, drying, calcination, sintering, and shaping) silica membranes were received.[28–30,37] They are denoted according to their respective pore system and mean pore size, for example, as 20 nm for a purely mesoporous material, or with 20–3500 nm (rounded) in the case of a meso-macroporous material. For monomodal pore systems, mean pore sizes in the range from 10 to 39 nm (meso) and from 270 to 3272 nm (macro) could be realized. As expected, specific surface areas (S\text{BET}) increased with decreasing pore diameter and ranged between 66 and 343 m² g⁻¹ (Table 1). The relatively high surface area of the macroporous material (66 m² g⁻¹) is attributed to a small amount of micropores that could not be completely removed during the sintering process. The fact that AChE is larger than 3 nm implies that these micropores become unaccessible and the material can be effectively described as macroporous. For supports with hierarchical pore structure, combinations of reaction pores (mesopores) in a range from 20 to 40 nm (see Figure S1, Supporting Information, for nitrogen physisorption measurements on sample 20–3500 nm) and larger transport pores (macropores) with a mean size from 360 to 3781 nm were realized. They have specific surface areas between 105 and 262 m² g⁻¹ (Table 1). To compare supports with monomodal and hierarchical pore structure regarding their performance as potential sensor component, the following representative samples have been selected: 20 nm (mono-meso), 20–3500 nm (meso-macro), and 3250 nm (mono-macro). Silica membranes were examined by scanning electron microscopy (SEM) (Figure S2, Supporting Information) and subjected to porosimetry (Table 1). The sol–gel process generates reproducible materials (±5% tolerance regarding mean pore diameter and pore volume) when using exactly the same procedure. The terminology for the preparation conditions is as follows: xg-P stands for the initial weight of PEO. A-7M-120-xh represents the conditions of the hydrothermal treatment in 7 M ammonia solution at 120 °C for x hours. Drying was carried out in an oven from overheated water (oH₂O). Abbreviations s and c denote sintering and calcination.

The shrinkage of the sol–gel materials is influenced by their synthesis conditions (e.g., ageing and drying) and their textural properties can therefore vary significantly. Consequently, larger cylindrical and crack-free monoliths were synthesized and shaped by further processing (core drilling and membrane sawing) into sol–gel membranes with defined geometry. The drying process is crucial for the preparation of crack-free silica monoliths, because unfavorable drying conditions can lead to complete degradation of the gel network. Stress on the network during drying is mainly caused by capillary tension. In addition, solvent removal leads to a decrease of the gel volume, referred to as shrinkage. Thereby occurring syneresis causes a stiffening of the gel network and the rigid network is prone to breaking. In their classical textbook, Brinker and Scherer describe the drying of porous gels in more detail.[38]

Evaporation occurs when the vapor pressure of the solvent p\text{v} is smaller than the equilibrium vapor pressure p\text{e}.
A tension $P$ develops in the solvent phase, which is related to the vapor pressure of the corresponding liquid according to the Gibbs–Thomson equation:

$$P = \frac{RT}{V_m} \ln \frac{p_v}{p_0}$$

where $R$ denotes the ideal gas constant, $T$ is the temperature, and $V_m$ the molar volume of the liquid. Figure 2 depicts the drying of a gel with cylindrical pores of radius $r_p$ schematically. At first, the gel-surrounding solvent evaporates (Figure 2A), while the liquid vapor interface is flat. In the case of a spreading liquid (with a contact angle between liquid and solid phase of $\theta = 0^\circ$), a thin film (of width $\delta$) remains on the surface of the solid. Afterward, the liquid in the pores starts to evaporate. If the contact angle between pore liquid and pore wall is $\theta < 90^\circ$, a concave meniscus is formed due to $P$, the described tension in the fluid (Figure 2B). $P$ depends on the interfacial energy between liquid and vapor phase $\gamma_{LV}$ and on the radius of the meniscus $r_m$. This relationship can be characterized by the Young–Laplace equation:

$$P = -\frac{2\gamma_{LV}}{r_m}$$

### Table 1. Preparation conditions and porosimetry data for selected monolithic silica samples.

| Sample      | Preparation conditions | $d_{macro}$ [nm] | $V_{macro}$ [cm$^3$ g$^{-1}$] | $d_{meso}$ [nm] | $V_{meso}$ [cm$^3$ g$^{-1}$] | $\epsilon_{total}$ | $S_{BET}$ [m$^2$ g$^{-1}$] |
|-------------|------------------------|------------------|-------------------------------|------------------|-------------------------------|---------------------|---------------------------|
| 20 nm       | 0g-P-A-7m-120-6h-OH$\text{H}_2$O  | —                | —                             | —                | —                             | 20                  | 1.02                      | 0.69                      | 343                       |
| 20–3500 nm  | 2.3g-P-A-7m-120-3h-OH$\text{H}_2$O-c | 3574             | 2.50                          | 20               | 0.93                          | 0.88                | 205                       | *Based on nitrogen physisorption measurements and MIP data.* |
| 3250 nm     | 2.0g-P-OH$\text{H}_2$O-s         | 3272             | 1.22                          | —                | —                             | 0.73                | 66                        | *Based on nitrogen physisorption measurements and MIP data.* |
The capillary tension draws the compliant network into the liquid and the occurring volume shrinkage is equal to the volume of evaporated liquid. This is referred to as the first stage of drying (constant-rate period).

\[ \gamma = \frac{2p}{r} \]

This point is critical and cracking most likely. From here onward, the first falling-rate period takes place, where the depth \( h \) of the meniscus recedes into the pores, still leaving a thin film of solvent on the surface (Figure 2C). Through this film, the solvent flows to the exterior surface constantly, where most evaporation takes place.

In this work, we introduce a cost- and time-efficient drying process together with an easy and safe experimental setup. For that purpose, the wet gel—placed in a closed plastic vessel—was covered with water (twofold volume of the monolith) to prevent partial drying during heating to 120 °C. It was subsequently dried at this temperature, that is, above the boiling point of water at normal pressure. The high temperature was important to minimize the interfacial energy of water.

Since evaporating water can leave the vessel only slowly through the small, defined puncture in the lid, the vapor pressure \( p_v \) should be kept close to its equilibrium value \( p_e \). This would decrease the evaporation rate as well as capillary tension according to the Gibbs–Thomson equation, cf. Equation (1).

2.2. Functionalization of the Silica Surface

Immobilization of AChE was performed through functionalization of the silica surface with 3-(glycidoxypropyl)dimethoxysilane. On the one hand, the monomethoxy modification of the silane prevents its polymerization, and on the other hand, the glycidoxy group binds the enzyme directly to the surface without needing an additional spacer, for example, glutaraldehyde. Concentrations between 0.3 and 100 vol% can be applied for surface functionalization with silanization reagents.

Preliminary studies have shown that the materials become hydrophobic after using a 3 or 5 wt% 3-(glycidoxypropyl)dimethoxysilane solution, preventing their use in aqueous medium (Table S1, Supporting Information). For this reason, and because pore-blocking should be avoided, a concentration of 1 wt% was used. Prior to functionalization, all materials were gently rehydroxylated in water for 3 h at 25 °C to achieve a high and comparable concentration of surface hydroxyl groups. The concentration of hydroxyl groups on the pore surface was calculated by thermogravimetric measurements. Based on the mass loss and taking into account the specific surface area, five hydroxyl groups per nm² could be generated with the rehydroxylation step. Diffuse reflectance
infrared Fourier transform spectroscopy was used to study the functionalization with 3-(glycidoxypropyl)dimethylmethoxy silane and associated changes in the surface properties. A rehydroxylated sample was used for comparison (Figure 3). Because of the deformation vibrations of the SiO$_2$ framework the fingerprint region in which, for example, ring deformation vibrations of the epoxy group of the functionalization reagent are found (950–815 cm$^{-1}$), could not be used for evidence. The spectrum of the rehydroxylated material provides a sharp band at 3743 cm$^{-1}$ (stretching vibrations of isolated hydroxyl groups). Based on its intensity only a small amount of water is physisorbed on the surface. The bands at 1768 and 1628 cm$^{-1}$ cannot be unambiguously assigned. They may represent deformation vibrations of the SiO$_2$ framework, as they are also present in the spectrum for the functionalized material. The successful binding of the functionalization reagent is demonstrated by the bands corresponding to the asymmetric and symmetric stretching vibrations of the $-$CH$_2$ groups of the propyl chain and the methyl groups of the silane (from 3100 to 2700 cm$^{-1}$). The sharp, intense band for the hydroxyl groups is now obscured by a broad band from physisorbed water (3700–3250 cm$^{-1}$). This qualitative proof is supported by the elemental analysis (Table S1, Supporting Information) and SEM-EDX images (Figure S3, Supporting Information).

Figure 3. DRIFT-IR spectrum of a freshly rehydroxylated, purely macroporous membrane (top) and after functionalization with a 1 wt% 3-(glycidoxypropyl)dimethylmethoxy silane solution (bottom).

Further, we determined the number of functional surface species ($N_{\text{glycidoxy}}$) quantitatively by titration (Table 2; Table S2, Supporting Information). Given values represent the mean from three individual investigations. It should be mentioned that for each membrane the same amount of modification reagent (corresponding to the same number of silane molecules) was used to ensure comparability. The data in Table 2 ($N_{\text{glycidoxy}}$ per nm$^2$) reveal that the offered 1 wt% 3-(glycidoxypropyl)dimethylmethoxy silane solution presents an overshoot for the macroporous membranes (sample 3250 nm) and an undershoot for the mesoporous membranes (sample 20 nm). The influence of the pore system can already be seen at this stage, as the macroporous sample contains significantly less functional groups per mass (255 µmol g$^{-1}$) than the mesoporous sample (419 µmol g$^{-1}$). The hierarchical support combines features of these two materials and demonstrates the positive effect of a meso-macroporous architecture by an intermediate concentration (392 µmol g$^{-1}$). As the same amount of silanization reagent has been employed for all samples, their area-normalized functionalization is inversely proportional to their $S_{\text{BET}}$-values. For example, $N_{\text{glycidoxy}}$ per nm$^2$ for sample 20–3500 nm is by a factor of 1.7 higher than for the mesoporous sample 20 nm and its specific surface area is smaller by nearly the same factor (cf. Table 2).

2.3. Impact of Pore Space Hierarchy on Transport Efficiency

By analyzing the maximum amount of immobilized AChE as a function of pore size and membrane morphology, the focus was on the reaction pores (mesopores). The determination of enzyme loading onto a support with the protein assay kit is based on a reduction of Cu$^{2+}$ to Cu$^+$ ions. Bicinchoninic acid was used to complex the monovalent copper ions. Extinction of the solutions (Table S3, Supporting Information) was determined at 562 nm and converted into enzyme loading ($c_{\text{AChE}}$) by calibration. The successful enzyme immobilization is confirmed by SEM-EDX measurements (Figure S3, Supporting Information).

For comparability, the determination of the maximum loading concentration ($c_{\text{max}}$) for AChE was carried out per membrane with fixed geometry, because it reflects envisioned operation of these materials as geometrical unit (with given dimensions) in a flow-through reactor or sensor design. Total porosities of these membranes expectedly varied according to the individual pore systems (cf. $\phi_{\text{total}}$ in Table 1). From the data in Table 3 it is clear that also $c_{\text{max}}$ (AChE) depends on the morphology of a pore system, because the surface area of a membrane ($S$) available for immobilization is intrinsically related to it. The analyzed data reveal that the amount of enzyme bound to a support ($c_{\text{max}}$, average of three individual measurements on different membranes for a given pore system) is less than the enzyme amount offered with the starting solutions ($c_{\text{theol}}$). Depending on the pore system and pore size, however, this only happens for higher theoretical concentrations (Table S3, Supporting Information). For the pore system containing only mesopores, the low loading can be attributed to pore blocking effects and associated obstruction of parts of the pore space. The mesopore network represents a tubular, 3D interconnected structure which—due to the size of the enzyme and bottle-necking effects—can impose severe steric hindrance and ultimately prevent the access of the enzyme to major parts of the surface. The simulation of hindered diffusion in physical reconstructions of the mesoporous skeleton from hierarchical,

Table 2. Number of functional groups ($N_{\text{glycidoxy}}$) on the support materials.

| Sample         | $S_{\text{BET}}$ [m$^2$ g$^{-1}$] | $N_{\text{glycidoxy}}$ [µmol g$^{-1}$] | $N_{\text{glycidoxy}}$ [nm$^{-2}$] |
|----------------|----------------------------------|---------------------------------------|-----------------------------------|
| 20 nm          | 343                              | 419 ± 6                               | 0.7 ± 0.01                        |
| 20–3500 nm     | 205                              | 392 ± 5                               | 1.2 ± 0.01                        |
| 3250 nm        | 66$^{\text{th}}$                 | 255 ± 4                               | 2.3 ± 0.03                        |

$^a$Calculated using Avogadro’s constant; $^b$Based on nitrogen physisorption measurements and MIP data.
meso-macroporous silica monoliths has revealed that only \( \approx 40\% \) of the mesoporosity (mean mesopore size: 20 nm) remains accessible for molecules with a size of 6 nm. \(^{59}\) This situation is similar to that considered here regarding mean mesopore size and size of the AChE molecules. Compared with the 20 nm sample (mono-meso), the 3250 nm sample (mono-macro) offers \( \approx 99\% \) smaller surface area (Table 3). Still, a \( c_{\text{max}} \) of 4.1 \( \mu \)g per membrane is achieved, corresponding to \( \approx 10\% \) of \( c_{\text{max}} \) for the mesoporous material (38.5 \( \mu \)g per membrane). Obviously, this originates in a quantitative exploitation of the macropore surface by the enzyme under conditions when steric hindrance is practically absent.

While a purely macroporous material offers excellent properties regarding liquid-phase mass transfer, that is, relatively unhindered diffusion of large molecules and also little obstructed, highly permeable flow (when used in a flow-through design)\(^{21,22}\) it can bind only much fewer enzyme molecules and advection-dominated mass transfer in the macropores with little hindrance and thereby fine-tune transport to and from the enzyme in the mesopores. Since enzyme immobilization is based on a reaction between surface epoxide groups and amino groups of amino acids in AChE, the immobilization process and trends observed for the individual pore systems should be similarly applicable to a number of other enzymes.

ACh is degraded by AChE into choline and acetic acid (Figure 4), so that the reaction can be monitored by potentiometric pH measurement. Enzyme activity strongly depends on the pH; beyond the pH optimum enzyme activity decreases up to complete activity loss and associated denaturation or a permanent damage of the enzyme.\(^{62}\) The pH optimum for AChE is in the range of 7–9, thus, a disodium hydrogen phosphate buffer solution was used to adjust the starting pH to 8.\(^{61}\) To determine the response time of a sensor membrane by pH changes from 8 to 7 as a function of ACh concentration and calculate apparent reaction rates, the experimental setup in Figure S4, Supporting Information, was held constant regarding all relevant aspects, for example, membrane geometry, solution volume, and stirring speed. Differences in response times could therefore be attributed to the impact of the individual pore systems. The response time is used to estimate an apparent reaction rate of the ACh degradation according to:

\[
\nu_{\text{app}} = \frac{n_{\text{HA}}}{t_{8-7}}
\]

In Equation (4), \( n_{\text{HA}} \) is the amount of formed acetic acid (back-calculated from the potentiometric pH measurements) and \( t_{8-7} \) denotes the response time for a pH change from 8 to 7. The degradation of ACh and the activity of free AChE were monitored in advance (Figure S5, Supporting Information).

Table 3. Maximum AChE loading concentrations (\( c_{\text{max}} \)) for membranes with identical geometry, but different pore space morphology and surface area \( S \).

| Sample          | \( S \) [m\(^2\) per membrane] | \( c_{\text{max}} \) (AChE) [\( \mu \)g per membrane] | \( c_{\text{max}} \) (AChE) [\( \mu \)g per membrane] |
|-----------------|---------------------------------|---------------------------------|---------------------------------|
| 20 nm           | 4.4\(^{a}\)                     | 60                              | 38.5 \( \pm \) 0.8              |
| 20–3500 nm      | 1.0\(^{a}\)                     | 40                              | 15.3 \( \pm \) 0.4              |
| 3250 nm         | 0.02\(^{a}\)                   | 40                              | 4.1 \( \pm \) 0.2               |

\(^{a}\)Based on nitrogen physisorption measurements; \(^{b}\)Based on the MIP data only.

Figure 4. AChE-catalyzed ACh degradation with attribution of the rate constants and resulting products.\(^{60,61}\)
With 4 µg of free AChE a reaction time of $t_{50-70} = 106\ s$ was found for the targeted pH change. Based on Equation (4), the reaction rate is 31 nmol s$^{-1}$, equivalent to an activity of 1.8 U ($=1.8\ \text{µmol min}^{-1}$), close to the specification by the manufacturer ($518\ \text{U mg}^{-1} \times 0.004\ \text{mg} = 2.1\ \text{U}$).

The impact of the pore system (morphology) on response time and apparent reaction rate of the developed model system was investigated at an AChE loading of 4.1 µg per membrane for all three pore systems, a value that reflects $c_{\text{max}}$ for the purely macroporous sample (cf. Table 3). That loading was chosen as a consequence of the conditions in this work and corresponds to the highest common loading in a comparison including all three pore systems. This means that the three materials (membranes) had the same amount of enzyme molecules (4.1 µg) distributed over their intrinsically different pore systems. Experimental results summarized in Table S4, Supporting Information, reveal that for materials with monomodal pore structure (mono-meso, mono-macro) the response times approach a maximum value (Figure S6, Supporting Information), close to the specification by the manufacturer (518 U mg$^{-1}$). Looking at the corresponding Lineweaver–Burk plots (Figure S7 and Table S5, Supporting Information), it should be emphasized that the recorded apparent kinetic data reflect both the intrinsic reaction kinetics and eventual transport limitations by an individual pore system. Figure 5 reveals only a slight disadvantage for the hierarchical pore system (20–3500 nm) compared to the macroporous structure (3250 nm), as seen in their close $v_{\text{max}}$-data (red circles). Clearly, transport into the macroporous membrane is least obstructed and its response is fastest. The purely mesoporous membrane shows a much slower response time (cf. Table S4, Supporting Information), indicating a substantially obstructed transport into and through its pore system, even though mesoporous and macroporous systems share similar porosities ($t_{\text{dry}} = 0.69$ and 0.73, Table I).

For the purely macroporous material the actual enzyme loading (also its maximum loading) is sufficient and transport through the macropore network least obstructed compared with the hierarchical and the purely mesoporous material. Consequently, its efficiency is also highest. On the other hand, the maximum enzyme loading of the purely macroporous membrane may not suffice in certain applications like the detection of pesticides in food products. For example, $\approx 12.9\ \text{µg AChE}$ per membrane are needed for the detection of the legally prescribed maximum residue level of the pesticide carbofuran.[66] These 12.9 µg of AChE (3.2 times higher than $c_{\text{max}}$ of the purely macroporous material) correspond to $\approx 34\%$ and $\approx 83\%$ of the maximum loadings for the mesoporous sample 20 nm and the hierarchical sample 20–3500 nm, respectively (cf. Table 3). Importantly, purely macroporous membranes with decreased macropore size and therefore larger macropore surface as well as higher $c_{\text{max}}$ compared to sample 3250 nm cannot achieve the targeted AChE loading of 12.9 µg (cf. Table S3, Supporting Information).

With results for 12.9 µg AChE per membrane (blue circles, Figure 5) the purely mesoporous material demonstrates an impact of the enzyme loading on the reaction rate. It can be attributed to faster substrate conversion due to a larger number of active centers and decreased diffusion lengths of substrate to the enzyme and of products out of the pore network. Also for the support with hierarchical structure reaction rates increase at higher loading, that is, $v_{\text{max}}$ is increased by a factor of 5 for the 3.2 times higher AChE loading (Figure 5). The beneficial effect of pore space hierarchy is clearly documented here—the hierarchical pore system responds more than twice as fast as the purely mesoporous material. A biosensor equipped with the hierarchical support can therefore demonstrate improved efficiency.

Looking ahead, these studies may be transferred to a flow-through design. Then, due to pressure-driven flow through the hierarchical material, transport in its macropores moves from diffusion-limited to advection-dominated and pure diffusion takes place only inside a thin mesoporous skeleton. This operation will also allow to move from the apparent to the intrinsic reaction kinetics (as transport limitations are removed) and access parameters like the reaction order, rate constants, and activation energies.[130] On the other hand, with an adequate enzyme loading in the macropores, a purely macroporous material may suffice as functional component in biosensor applications and at the same time take full advantage of the fast mass transfer due to forced advection in a flow-through design. If required, the surface area (and thus, enzyme loading) of the macropores can be increased to some extent by reducing the macropore size and increasing the macroporosity.[122]

3. Conclusions
The effect of a hierarchical pore system on the diffusive response of sol–gel based materials was systematically studied.
and compared with corresponding monomodal (purely mesoporous and macroporous) pore systems for the first time. Supports in the form of membranes (6 mm-diameter × 0.5 mm thickness) were prepared by the sol–gel process to address the impact of the three different pore systems on features of the functional components derived by subsequent enzyme immobilization. A general preparation scheme was adapted with varying composition of the starting synthesis mixture as well as various additional treatments such as sintering and alkaline ageing. Mesoporous materials have been prepared as stable, shaped bodies by a novel drying method. Hierarchical systems were prepared via polymer-induced phase separation with mesopores and macropores from the same size ranges as realized for the monomodal pore systems. All surfaces were functionalized with 3-(glycidoxypropyl)dimethylmethoxysilane and AChE was then immobilized covalently via the epoxide group of the silane. The immobilized enzyme amounts depended on the specific surface area of the materials. Membrane preparation, surface functionalization, and enzyme immobilization were realized in good reproducibility, which underlines the suitability of the adapted synthesis strategy.

It has been shown that a membrane with hierarchical pore system and a macropore size similar to a purely macroporous support exhibits a much higher AChE loading capacity due to the additional mesopores. Further, it was investigated if the hierarchy has an impact on the response time of AChE-based functional membranes. At this stage, studies remained limited to purely diffusive membrane transport, so that apparent reaction rates reflected intrinsic diffusion limitations of a pore system in addition to the intrinsic reaction kinetics. Response times of the prepared membranes were determined for the AChE-catalyzed degradation of ACh to choline and acetic acid. With 4.1 μg AChE per membrane the purely macroporous material revealed a slight advantage over the hierarchical pore system; exclusive transport in the macropores is least obstructed giving the fastest response. On the other hand, for the detection of the legal residue level of carbofuran ≈12.9 μg AChE per membrane are required (exceeding the cmin of the purely macroporous material). Then, the hierarchical pore system gives the fastest response by combining advantages of the two monomodal systems, that is, enhanced mass transfer and higher enzyme loading.

Gained insight will help to tailor new hybrid materials based on a functional component and an inorganic support, for example, in chemo- and biosensor technology to improve sensor performance. The gain could be particularly impressive as the application is realized in a flow-through design and full advantage is taken of both a highly permeable (flow-through) macropore system and negligible resistance to diffusion through a thin (just ≈1 μm thick) mesoporous skeleton in a hierarchical support.

4. Experimental Section

Chemicals: TEOS (99%) came from abcr (Karlsruhe, Germany). AChCl (99%), bromothymol blue, and PEO (average molecular weight: 100 000) were received from Alfa Aesar (Haverhill, MA). Sulfuric acid (93%) came from VWR International (Darmstadt, Germany). Ammonium hydroxide solution (25%) and hydrochloric acid (37%) were purchased from AppliChem (Darmstadt, Germany). Potassium bromide (IR-grade), n-butylamine (97%), and sodium dihydrogen phosphate dihydrate came from Merck (Darmstadt, Germany). 3-(glycidoxypropyl)dimethylmethoxysilane (97%), AChE (electrophorus electricus, 518 U mg⁻¹), ethanol (analytical standard), and disodium hydrogen phosphate were obtained from Sigma-Aldrich (St. Louis, MO). Chemicals were used without further purification.

Support Synthesis: For the sol–gel based synthesis of the materials with different pore systems (cf. Figure 1) the following molar ratios of starting components had been employed: 1.0 mol H₂O : 0.025 mol H₂SO₄ · x mol PEO : 0.04 mol TEOS.

First, sulfuric acid (2.54 g) and PEO (0–3 g) were added to distilled water (18 g) under vigorous stirring. After stirring for 30 min at room temperature, the reaction mixture was cooled with an ice bath to 4 °C and TEOS (14.6 g) was added. When (after an additional 30 min of vigorous stirring) solutions became transparent, each mixture was poured into a 15 mL centrifugal tube. Before gelation, the mixtures were evacuated (2 min, 0.1 bar) to remove air bubbles. Gelation was performed in an oven for 24–72 h at 50 °C under helium atmosphere at constant pressure of 7 bar. After cooling, the wet gels were washed for 18 h with distilled water to neutral pH. Pore widening of mesoporous and hierarchical materials was carried out in small lab autoclaves with solid–solvent ratio of 1:10 at 120 °C/180 °C for 3–48 h using 7 m ammonia solution. A washing step with distilled water (18 h) was added. Hierarchical materials were dried at room temperature for 1 day. Samples with monomodal pore structure were placed into their starting plastic vessels and covered with water. The centrifugal tubes were closed, the lid provided with a leak, and the materials dried in an oven at 120 °C for 12 h. Samples with organic residues were calcined for 4 h using a heating rate of 3 °C min⁻¹ from room temperature up to 550 °C. Macroporous materials were additionally sintered at 900 °C for 1 h in a muffle furnace (Nabetherm, Lilienthal, Germany) with a heating rate of 3 °C min⁻¹ to remove any mesoporosity. Using a core drill (Guede, Wolpertshausen, Germany) and a SAW 15 from Logitech (Lausanne, Switzerland) membranes with a diameter of 6 mm and a thickness of 0.5 mm were prepared.

Surface Modification and Enzyme Immobilization: Before surface functionalization, samples were rehydroxylated in water (3 h, room temperature) and dried in an oven at 120 °C. Activated membranes were mixed with a fresh 3-(glycidoxypropyl)dimethylmethoxysilane solution (1 wt%) in a polypropylene vessel at a ratio of 1:20. A 14 v/v water/ethanol mixture was used as solvent for the silane solution. Reaction took place at 60 °C in a water bath under constant stirring for 6 h. After washing the membranes for 5 min with solvent under shaking, a drying step for the post-condensation of physisorbed molecules was carried out at 120 °C for 2 h. The membranes were finally washed with solvent three times for 10 min and dried at 120 °C for 2 h.

For enzyme immobilization, each functionalized membrane was transferred into a 2 mL reaction vessel and mixed with 150 μL enzyme buffer solution in an ice bath at 0 °C. The enzyme concentration varied from 4 to 60 μg mL⁻¹. To ensure complete filling of the pore system, reaction vessels were placed in an ice bath in a vacuum oven and evacuated for 15 min at 0.1 bar. Subsequently, immobilization took place in a closed vessel at 4 °C in the refrigerator for 16 h. After washing each membrane three times for 5 min with 500 μL buffer solution, the membranes were stored in the reaction vessels (covered with 50 μL buffer solution) in the refrigerator at 4 °C. A 0.01 M phosphate buffer (pH 8) was used as buffer solution. All materials and solutions used for enzyme immobilization were autoclaved at 120 °C. Although freshly prepared samples were worked upon, the final membranes with immobilized enzyme could still be used after a few months when properly stored at 4 °C in the buffer solution under exclusion of light. With these conditions, an activity loss of ≈10% was observed after 2 months.

Characterization: Mercury intrusion porosimetry was run on Poremaster (Quantachrome Instruments, Boynton Beach, FL) from 0.15 to 400 MPa. Pore size distributions were derived from the MIP data with Quantachrome software according to the Washburn equation, setting the mercury contact angle to 140°. The measured pressure range corresponds...
to pore diameters between 3.5 and 10 μm. Nitrogen physisorption data were acquired on an ASAP 2000 (Micromeritics, Norcross, GA). Prior to the measurements, samples were activated for 24 h at 120 °C under vacuum (10−3 bar). Specific surface areas were calculated using the Brunauer–Emmett–Teller equation (p/p0 = 0.05–0.35). Silica membranes were examined under a Leo Gemini 1530 scanning electron microscope (Zeiss, Oberkochen, Germany), for which Au was vapor-deposited onto sample pieces at 10 kV. Elemental analysis was conducted on a vario Micro cube (Elementar, Langenselbold, Germany).

To verify functionalization, DRIFT spectra were recorded on a Vector 22 (Bruker, Billerica, MA) from 4000 to 1500 cm−1. Prior to the measurements, samples were ground (to receive a particle size < 5 μm) and diluted with potassium bromide to reduce absorption. For quantitative analysis, 500 mg sample were mixed with 25 mL of 0.01 M n-butylamine solution for 1 h at 60 °C and titrated by dropwise addition of 0.01 M HCl (indicator: bromothymol blue). The number of functional groups per n-butylamine was calculated from the sample mass and required volume of hydrochloric acid. The epoxide groups reacted with n-butylamine under ring opening and remaining n-butylamine was back-titrated with HCl (see Supporting Information for further details).

To derive enzyme concentration in the reaction solutions before and after immobilization, spectrophotometric studies were conducted using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) and a UV-1600 PC spectrophotometer from VWR International (Darmstadt, Germany) at 562 nm. Potentiometric pH analysis of AChE-catalyzed ACh degradation was performed with the pH electrode InLab Ultra Micro pH and the measuring device Seven Easy pH from Mettler Toledo (Zurich, Switzerland), for which Au was vapor-deposited onto 250 μL solution. All solutions for the measurements were tempered to 25 °C. The general procedure and more detailed information on the potentiometric analysis can be found in the Supporting Information (Table S6, Supporting Information).

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

drying process, enzyme immobilization, meso- and macroporous materials, pore space morphology, response time

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