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Impedance characterization of biocompatible hydrogel suitable for biomimetic lipid membrane applications

**Agnieszka Mech-Dorosz^{1,2}, Muhammad Salman Khan¹, Ramona Valentina Mateiu³,
Claus Hélix-Nielsen^{4,5}, Jenny Emnéus¹, Arto Heiskanen^{1,*}**

¹Technical University of Denmark, Department of Biotechnology and Biomedicine, Produktionstorvet, Building 423, DK-2800 Kgs. Lyngby, Denmark

²Present address: Novo Nordisk A/S, Brennum Park 24 K, 3400 Hillerød, Denmark

³Technical University of Denmark, Center for Electron Nanoscopy, Fysikvej, Building 307, 2800 Kgs. Lyngby, Denmark

⁴Technical University of Denmark, Department of Environmental Engineering, Bygningstorvet, Building 115, DK-2800 Kgs. Lyngby, Denmark

⁵Aquaporin A/S, Nymøllevej 78, DK-2800 Kgs. Lyngby, Denmark

*Corresponding Author

Phone: +45-45-256839

e-mail: arhe@dtu.dk

Abstract

Hydrogels, biocompatible and hydrophilic polymeric networks, have been widely applied in, e.g., pharmaceutical and biomedical research. Their physico-chemical properties can be fine-tuned by changing the fraction and molecular structure of cross-linkers. Hydrogel layers with varying thickness have also been used to support biomimetic lipid bilayers on microfabricated electrodes for studies using electrochemical impedance spectroscopy (EIS). To provide deeper understanding of the impedimetric behavior of thick hydrogels that are covalently tethered on microfabricated electrodes and the influence of cross-linking, we present here a thorough EIS characterization of poly(2-hydroxyethyl methacrylate) hydrogels cross-linked with poly(ethylene glycol)-dimethacrylate in ratios 1:100, 1:200, and 1:400. We propose an equivalent circuit model comprising an open-boundary finite-length Warburg element and constant phase element in series to describe the mass transfer differences between the bulk hydrogel and covalently tethered domain at the electrode-hydrogel interface. The results indicated that an increased fraction of the hydrophilic high-molecular weight cross-linker significantly decreased the charge transfer resistance for hexacyanoferrate(III/II), which could be attributed to increased permeability and decreased electrode passivation due to the lower degree of tethering on the acrylate modified electrodes. Cryo-SEM visualization of the structural differences caused by cross-linking showed good agreement with the EIS results, whereas the degree of hydration of the hydrogels did not show any statistically significant differences.

Keywords: Impedance spectroscopy; Poly(2-hydroxyethyl methacrylate) hydrogel; Poly(ethylene glycol)-dimethacrylate cross-linker; Covalent tethering; Cryo-SEM

1. Introduction

Since the pioneering work of Wichterle and Lim in 1960 [1], hydrogels, i.e. three dimensional cross-linked polymer structures with an ability to imbibe large amounts of water, have attracted great attention due to their excellent biocompatibility. Hydrogels have been increasingly recognized as materials in a wide spectrum of biomedical applications ranging from tissue engineering [2] and drug delivery systems [3–9] to construction of contact lenses [10,11], biosensor devices [12–14] and biomimetic systems, where they can function as porous supports for lipid membranes [15–20] and lipo-polymersomes [21], as well as substrates mimicking cellular cytoskeleton [22] or extracellular matrix (ECM) [23,24].

Sensitivity of hydrogels to different physical and chemical factors, e.g., temperature, potential, electrolyte concentration, pH, and degree of hydration, make them excellent "stimuli responsive" or "smart" materials for construction of chemical sensors and biosensors [25]. Aside from utilization as a passive immobilization matrix, their swelling capacity can facilitate construction of responsive matrices dependent on environmental changes. Due to elastic modulus comparable to that of living tissues and excellent biocompatibility, hydrogels derived from poly(2-hydroxyethyl methacrylate) (pHEMA) are among the most widely studied hydrogels and the first ones with commercial biomedical applications [26,27]. Similarly, poly(ethylene glycol) (PEG) and its acrylate derivatives are widely utilized in biomedical applications due to their good biocompatibility and non-immunogenicity. Flexibility in PEG hydrogel formulation makes it a primary choice for constructing porous scaffolds similar to certain type of soft tissues [24]. Modified PEG macromers have been successfully utilized as mimic of ECM [23,24] and for constructing biodegradable scaffolds for controlled drug release [3,4,7].

The performance of a hydrogel in a particular application is dependent on its molecular network structure usually characterized by several parameters, including polymer volume fraction in the swollen state, molecular weight of the polymer chain between two cross-linking points (cross-linking density) and the corresponding mesh size, which determines the maximum diameter of the molecules and ions that can pass through the hydrogel network [4]. Polymer volume fraction in the swollen state determines the amount of fluids that may be absorbed and retained by the hydrogel, which reaches equilibrium with its surroundings after polymerization. Swelling capacity of hydrogels is also exceptionally important since it contributes to their *in vivo* and *in vitro* biocompatibility [4]. HEMA monomers may be readily copolymerized with other acrylate, methacrylate and acrylamide monomers including PEG derivatives, which may serve as cross-linkers [28]. Copolymerized HEMA and poly(ethylene glycol)-dimethacrylate (PEG-DMA) monomers have found use in drug delivery systems [8,9], biosensor devices [12–14], and as solid support for stabilizing biomimetic lipid membranes [18–20] and lipo-polymersomes [21].

Electrochemical impedance spectroscopy (EIS) has been widely applied to probe both the resistive and capacitive properties of modified electrodes. Applications range from characterization of porous [29] and hydrogel coated [13,17,20,21,30] electrodes to detection in biosensing [31]. EIS has also been used in applications of biomimetic lipid membranes either directly on an electrode surface or on a supporting cushion layer (e.g. a hydrogel). Hence, a deeper understanding of the impedimetric behavior of hydrogels is important. For electrochemical characterization and applications of hydrogels, the passage of ions through the hydrogel and its thickness as well as placement and geometry of the electrodes are key factors [13,30,32,33]. To characterize the material properties of hydrogels with varying composition, a general approach has been to insert a hydrogel disc as a divider between two electrolyte filled chambers, each having an electrode to allow EIS measurements. Using such electrode

placement, the performed measurements focus on the changes in the membrane resistance rendered by, for instance, different cross-linkers and charged monomers [32,33]. Many applications rely on usage of lithographically patterned electrode microchips modified with a hydrogel. Depending on the degree of cross-linking and thickness of the used hydrogel, the required equivalent circuit model may vary to a great extent. Justin et al. reported on the EIS behavior of 6 μm thick pHEMA/PEGMA hydrogel films covalently immobilized on gold microdisc electrodes [13]. Yang et al. characterized complex 5 μm hydrogel covalently immobilized on various designs of interdigitated electrodes [30]. Kibrom et al. applied EIS to characterize 60 nm thick hydrogel supported protein-tethered lipid bilayer membrane suitable for functional protein studies [17]. In our previous work, pHEMA hydrogel has been used as a support for black lipid membranes (thickness 500 μm) [18,20] and lipo-polymersomes (in combination with a polyethersulfone membrane) [21]. For such applications, the hydrogel was composed of 1:200 molar ratio of PEG-DMA/HEMA monomers. EIS was applied to characterize the influence of the covalently immobilized hydrogel on the electrode interface impedance [20,21].

Here, we present a detailed EIS characterization of 1 mm thick pHEMA hydrogels that were covalently tethered on the working electrodes and the surrounding microchip surface. The study evaluated the influence of PEG-DMA cross-linking ratio (1:100, 1:200, 1:400) on mass and charge transfer behavior of electroactive species. To provide a simple and fast analysis of EIS results in hydrogel applications requiring a wide frequency range, we developed an equivalent circuit model that highlights the structural difference between the covalently tethered domain close to the electrode surface and the bulk hydrogel. The presented EIS characterization was correlated to the structural properties of hydrogels with different cross-linking ratios visualized

using cryo-SEM as well as to their swelling capacity based on dry and wet weight determination.

2. Experimental section

2.1 Chemicals

Poly(ethylene glycol)-dimethacrylate (PEG-DMA, Mw 1000 g/mol) was purchased from Polysciences, Inc. (Warrington, PA, USA). 2-hydroxyethyl methacrylate (HEMA), 1,4-butanedioldiacrylate (BDDA), ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), β -mercaptoethanol, 3-(trimethoxysilyl propyl) methacrylate (TMS-PMA), 30 % hydrogen peroxide, potassium hydroxide, acetic acid, potassium hexacyanoferrate(III) ($[\text{Fe}(\text{CN})_6]^{3-}$), potassium hexacyanoferrate(II) ($[\text{Fe}(\text{CN})_6]^{4-}$), and phosphate buffered saline (PBS; pH 7.4) were from Sigma-Aldrich Corporation (St. Louis, MO, USA). All aqueous solutions were prepared using ultrapure water (resistivity 18.2 M Ω ·cm) from a Mili-Q[®] water purification system (Milipore Corporation, Bedford, MA, USA).

2.2 Fabrication and surface modification of electrode microchips

Electrode microchips (Fig. 1A), comprising three working electrodes (WE), one counter electrode (CE), one reference electrode (RE), connecting leads and contact pads, were fabricated according to a previously published protocol through standard lithographic process using wet-oxidized 500 μm thick 4-inch silicon wafers (one side polished) [20]. The active electrode areas and contact pads on the patterned metal structures (10 nm Ti adhesion layer and 150 nm Au) were opened by reactive ion etching of the 500 nm thick silicon nitride passivation

layer deposited through plasma-enhanced chemical vapor deposition. Prior to use, the microchips were cleaned for 10 min in a mixture of H₂O₂ (25% v/v) and KOH (50 mM) followed by a potential sweep from -200 mV to -1200 mV in 50 mM KOH [34,35] using the on-chip three electrode set-up. To achieve covalent adhesion between the hydrogel and the gold electrode microchips, these were chemically modified. The chips were treated with 3% (v/v) H₂O₂ (1 h) to form a uniform layer of hydroxyl groups on the silicon nitride followed by formation of a self-assembled β -mercaptoethanol monolayer (1 h in 200 mM aqueous solution) on the gold surfaces. The hydroxyl groups were further functionalized by 1-h incubation in a 2 % (v/v) aqueous solution of TMS-PMA at pH 4 (adjusted using 1 M acetic acid) [20]. After each modification step, the microchips were rinsed thoroughly with water.

(Fig. 1)

2.3 In situ hydrogel polymerization

Each silanized gold electrode microchip was placed in a Teflon mold (schematically shown in Fig. 1B) for *in situ* polymerization of hydrogel. The mold diameter (6 mm) defined the polymerized hydrogel in such a way that the WEs were fully covered, whereas the CE and RE remained outside the hydrogel. The polymerization solution (diluted with water) contained PEG-DMA (30, 15, or 7.5 mM) and HEMA monomers (3 M) to obtain the molar ratios 1:100, 1:200, or 1:400, respectively, as well as BDDA (17 mM). Polymerization was induced by adding an aliquot of an aqueous solution containing the initiator (APS) and radical source (TEMED) to obtain the final concentrations of 27 mM and 33 mM, respectively. The resulting solution was thoroughly vortexed for 10 s, after which 28.3 μ l were introduced on the microchip assembled in the mold to obtain 1 mm thick hydrogel disc covering the WEs (Fig. 1C).

2.4 EIS characterization

The electrochemical cell for EIS characterization (Supplementary Material, Fig. S1) was filled with 300 μl of PBS containing 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (1:1). The microchips were characterized after each step of chemical modification and hydrogel polymerization using the on-chip CE. The sinusoidal perturbation potential (10 mV_{rms}) was applied with respect to the open circuit potential in the frequency range between 100 mHz and 1 MHz. EIS analysis was performed using a computer-controlled Reference 600 potentiostat from Gamry Instruments (Warminster, PA, USA) operated by EIS300 software (v. 6.10). Data analysis was done using EchemAnalyst software (v. 6.10) from Gamry Instruments by fitting the data to the equivalent circuit models using nonlinear least-squares (NLLS) regression.

2.5 Finite element simulations

Finite element (FE) simulation of electric field penetration through the covalently tethered hydrogel covering the electrodes was performed using COMSOL Multiphysics v.5.0. Two different cases were modeled in COMSOL Multiphysics: 1) The hydrogel only covered the WE while the CE was outside the hydrogel (the case experimentally characterized in the presented work); 2) the hydrogel covered both the WE and CE. 3D space dimension model along with frequency domain and time dependent study was selected in COMSOL. The models were constructed as shown in Fig. 2A-1 (case 1) and Fig. 2A-2 (case 2). The hydrogel thickness for each of the cases was 1 mm to mimic the experimentally studied hydrogel modifications. The AC/DC physics interface was selected to model electric field lines at various frequencies (100 mHz, 10 Hz, 1 kHz, 10 kHz, 100 kHz and 1 MHz) for both cases. The conductivity of PBS and pHEMA were defined based on previously published values as 1.6 S/m [36] and 3.5 mS/m [37],

respectively. The mesh consisted of tetrahedral elements. The characteristic values representing the mesh quantity and quality for both cases are shown in the Supplementary Material Table S3.

2.6 Cryo-SEM imaging

Cryo-SEM imaging was performed using an extended low-vacuum Quanta 200F FEG SEM (FEI Company, Hillsboro, OR, USA) with a Quorum PP2200 cryo-attachment (Quorum Technologies Ltd., East Sussex, UK). The samples were first cut into small pieces with a pair of scissors followed by mechanical fixing on an aluminum cryo-stub. Then, the samples were frozen by plunging them into nitrogen slush on the Quorum preparation bench and transferred to the cryo-preparation chamber, where they were freeze-fractured. The samples were not coated. For imaging, each sample was transferred onto the cold stage in the SEM chamber and sublimated at -90 °C for 15 min prior to imaging. The imaging was performed at -140 °C using an Everhart-Thornley (ETD) detector with an electron beam accelerated to 2 keV and a spot size of 2 nm with a 30 µm mechanical aperture. Quantification of sample morphology was done by manual size measurements using Image J software.

2.7 Determination of the degree of hydration

The swelling capacity of the hydrogels with different molar ratios of PEG-DMA/HEMA monomers was determined as the degree of hydration using the equation $\text{Hydration\%} = [(w_{\text{wet}} - w_{\text{dry}})/w_{\text{wet}}] \times 100\%$ [33,38]. The results are presented as average $\pm$ standard deviation, $n = 4$. The used hydrogel discs were polymerized on unmodified silicon nitride surfaces, from which they could be easily removed after polymerization. The w_{dry} determination was performed after

overnight drying of the hydrogels in a convection oven at 50 °C, while the w_{wet} determination was done after overnight soaking in PBS. Prior to the wet weight determination, the excess of PBS was removed from the hydrogel discs by quick blotting with tissue paper.

3. Results and discussion

Successful hydrogel modification of a microchips requires that the electrolyte, and hence, the electroactive species have access to the surface of the WEs only through the *in situ* polymerized hydrogel, i.e. no electrolyte flux should take place directly to the electrodes. We have previously demonstrated that this is achieved by covalent tethering of the hydrogel (primarily composed of pHEMA cross-linked with PEG-DMA) on both the gold WEs and surrounding silicon nitride that have been chemically modified [20,21]. EIS characterization of the chemical modification steps is shown in the Supplementary Material Fig. S2.

3.1 Characterization of 1:200 cross-linked hydrogel (PEG-DMA/HEMA)

In our previous studies, cross-linking ratio (PEG-DMA/HEMA) 1:200 was used for applications with biomimetic lipid membranes [20] and lipo-polymersomes (in combination with a polyethersulfone membrane) [21]. Hence, it is presented here as the standard hydrogel to compare the behavior of unconditioned and differently cross-linked hydrogels (PEG-DMA/HEMA – 1:100 and 1:400).

In the abovementioned hydrogel applications, EIS characterization was performed from 1 MHz down to 1 Hz and 100 mHz, respectively. When performing EIS characterization in such a wide frequency window using electrodes covered by a complex hydrogel matrix, it is crucial that the

electric field generated by the applied sinusoidal potential fully penetrates the hydrogel at each of the frequencies. Otherwise, the recorded spectral features, requiring a detailed equivalent circuit model, may not be a representative description of the tethered hydrogel. We performed FE simulations using COMSOL Multiphysics to evaluate the penetration of the electric field through a hydrogel matrix at different frequencies between 100 mHz and 1 MHz. Two different cases were modeled in COMSOL Multiphysics: 1) The hydrogel only covered the WE while the CE was outside the hydrogel (Fig. 2A-1; the case experimentally characterized in the presented work); 2) the hydrogel covered both the WE and CE (Fig. 2A-2). Fig. 2B-D shows electric field line representation of the FE simulations for three frequencies, 100 mHz, 1 kHz, and 1 MHz, respectively. More detailed presentation of the electric field behavior is shown in the Supplementary Material Fig. S3. The obtained simulation results clearly indicate that in case 1 (only the WE was covered) majority of the electric field lines at each of the simulated frequencies fully penetrated the hydrogel construct prior to reaching the CE. Due to the geometry and positioning of the CE and WE, primarily the electric field lines reaching the ends of the CE had tendency to pass through the side of the hydrogel construct instead of fully penetrating through the entire thickness of the hydrogel. In case 2, on the other hand, a significant portion of the electric field lines passed from the WE to the CE inside the hydrogel construct never penetrating it. In conclusion, based on the performed FE simulations, the entire frequency range (from 1 MHz down to 100 mHz) that was used in the experimental work presented here provides spectral information that is characteristic of the hydrogel behavior and its influence on the electrode interface impedance.

Fig. 2

3.1.1 Behavior after electrolyte conditioning

Usually after polymerization, a hydrogel is conditioned in an electrolyte solution prior to performing electrochemical analysis in order to equilibrate the electrolyte composition in the bulk hydrogel [20,30]. During conditioning, a hydrogel reaches its maximal inherent swelling capacity, which contributes to its permeability to low molecular compounds [39]. Our preliminary tests indicated that at least 16-h conditioning was required to reach constant EIS behavior. Hence, EIS characterization of conditioned hydrogels was performed after 24 h.

Fig. 3A shows two typical impedance spectra (presented as Nyquist plots) for 1 mm thick hydrogel. Each Nyquist plot consists of three characteristic parts: two semicircles (one in the high-frequency range and another in the intermediary frequency range) and a line in the low-frequency range. The semicircle in the high-frequency range is shown in the zoom-in view of the inset. Although the polymerization conditions were kept constant, the spectral features of several characterized hydrogel coated electrodes varied. These observed differences may result from the complexity of the formed porosity during the polymerization process shown in the cryo-SEM image of Fig. 3B. The phenomenon is analogous to the behavior of porous electrodes, where varying pore geometry significantly influences the acquired impedance spectra [40]. Although these hydrogels are not conductive like porous electrodes, the covalent tethering makes them an integral part of the electrode interface impedance. Hence, a variety of randomly structured pores with different dimensions and distribution may contribute to the characteristic impedimetric behavior. In our previous application on pHEMA supported black lipid membranes (BLMs) [20], we observed variation in the specific membrane capacitance and resistance of the BLMs. This could partially be attributed to the structural variation of the polymerized pHEMA.

Fig. 3

The solid lines in Fig. 3A indicate NLLS fitting of the data to the equivalent circuit model shown in Scheme 1. The semicircle in the high-frequency range is represented by the additional time constant (parallel element $R_{\text{par,hg}}$ and $C_{\text{par,hg}}$). Based on the observation of such a semicircle in previous studies on hydrogel coated electrodes and ultrafiltration membranes, it has been assigned to the capacitive and resistive properties of the bulk material, allowing modelling as a parallel RC element [17,20,30,41]. In some applications, a high-frequency semicircle has also been modelled using a capacitor in parallel with the rest of the equivalent circuit, described as a geometric capacitance (C_g) [42]. It has been attributed to different system/material properties depending on the scope of the presented application, e.g., membrane capacitance [43], coating capacitance for conducting polymer tube modification [44], and capacitance of interdigitated electrodes [45]. However, based on our evaluation, the high-frequency semicircle in spectra for thick hydrogels on WEs could not be described by C_g . Performed EIS simulations using C_g in the equivalent circuit generated spectra with an incomplete semicircle. In other words, to completely resolve such a high-frequency semicircle, the frequency range should have been extended beyond 1 MHz.

Scheme 1

The semicircle and line in the intermediary and low-frequency range are characteristic of the behavior described by Randles equivalent circuit [46]. The slightly recessed semicircle could be represented by a resistor and constant phase element (CPE, see Supplementary Material S4 for the mathematical description and Fig. S4-1 for the schematic illustration) in parallel [33]. This corresponds to the interface impedance of the underlying gold electrode, accounting for faradaic processes of the electroactive probe (charge transfer resistance, R_{ct}) as well as double

layer capacitance and surface inhomogeneity (CPE_{dl}). The slope of the low-frequency line is clearly greater than 45° , which is known as the characteristic feature of the semi-infinite diffusion impedance (Warburg impedance element, Z_w , see Supplementary Material S4 for the mathematical description and Fig. S4-2 for the schematic illustration) describing the behavior of an electrode-electrolyte interface where the diffusion layer thickness is not limited. Using Z_w complete fitting was obtained only down to frequencies between 1 and 10 Hz (Supplementary Material Fig. S5A). Although the presented frequency range may be narrowed in many applications in order to facilitate fitting using a simple equivalent circuit model, it would not be plausible in the case of the spectra acquired for thick hydrogels covalently tethered in the electrode surface since truncation of the spectra would eliminate the entire line corresponding to the diffusion impedance. Moreover, considering applications relying on EIS detection, such as monitoring of valinomycin in hydrogel supported lipid bilayers requiring spectral analysis down to 1 Hz [20], it is necessary to achieve a detailed understanding of the impedimetric behavior of hydrogels down to low frequencies. Instead of covalent tethering of *in situ* polymerized hydrogels, EIS based characterization of hydrogels has also been performed by placing the hydrogel between two chambers filled with electrolyte [32,33]. In this approach, the hydrogel is uniform throughout the entire thickness, allowing application of a simplified equivalent circuit model [32,33] that may be based on the general format of Randles equivalent circuit model [46]. As further discussed below, due to the covalent tethering of the hydrogel on the electrode and surrounding silicon nitride surface, two distinct domains are formed requiring more complex modeling of the mass transfer impedance behavior.

Studies on, e.g., polymer-coated electrodes and ion exchange or ion-selective membranes, have warranted description of the low-frequency impedimetric behavior using finite diffusion layer models [47,48]. The two known cases are the open-boundary (O element, Z_{wo}) and blocked-

boundary (T element, Z_{WT}) finite-length Warburg impedance (see Supplementary Material S4 for the mathematical description). In both cases, the initial slope of the Warburg line is 45° . At a low-frequency inflection point, Z_{WO} bends toward the Z_{Real} axis, whereas Z_{WT} turns into a 90° capacitive line (see the schematic illustration in Supplementary Material Fig. S4-3 and S4-4, respectively). The low-frequency behavior of the Nyquist plots in Fig. 3A is intermediary between that of W_O and W_T . Similar spectral behavior has been theoretically analyzed by Bisquert et al. [47], indicating the need of a CPE to describe the impedimetric behavior in the lowest end of the frequency range. As shown by the NLLS fitting in Fig. 3A, the series combination of Z_{WO} and CPE_{mtd} , the latter component accounting for deviation from the pure Z_{WO} behavior at very low-frequencies, provides a good description of the impedimetric behavior down to the lowest frequency end (the range for different parameter values obtained by analyzing several hydrogel samples is shown in Table 1). Fitting of the Nyquist plots using an equivalent circuit having Z_{WO} alone (Supplementary Material Fig. S5B) only gives a marginally better result than using Z_W alone (Supplementary Material Fig. S5A). Although Z_{WO} could not alone describe the behavior of the data, its significance as the Warburg element for finite diffusion layer behavior was demonstrated by using an equivalent circuit with Z_{WT} (instead of Z_{WO}) in combination with CPE_{mtd} . The result indicated that in the lowest end of the frequency range the slope of the generated Warburg line was consistently higher than shown by the data (Supplementary Material Fig. S5C).

Table 1

The requirement of two components to describe the mass transfer behavior of thick covalently tethered hydrogels can be plausibly explained by considering a hydrogel as a porous membrane of two structural domains with different mobility of ions. The upper domain, i.e. the bulk of the hydrogel, is interfaced on one side to the bulk electrolyte and on the other side to a more densely

packed hydrogel (the lower domain) covalently tethered to the microchip. The denser structure of the covalently tethered domain originates from the HEMA monomers coupled to the acrylate functionalities on the chip surfaces making the hydrogel less flexible to swell than the bulk hydrogel. The membrane potential of porous membranes comprises Donnan potentials, arising from separation of ions according to their charges, and a diffusion potential due to differences in ion mobility [41]. Considering ionic dimensions, cross-linked pHEMA hydrogels are highly porous, only causing a partial Donnan exclusion due to the increased electron density of the oxygen functionalities. Hence, the diffusion potential may be the predominating as the contribution to mass transfer impedance. In the case presented here, the diffusion potential originates from the two structurally different domains, clearly requiring the combination of two components in the equivalent circuit model, which is analogous to two-layer polymer films on electrodes presented by Freger [48].

3.1.2 Behavior immediately after polymerization

Fig. 4 shows a typical Nyquist plot for a hydrogel characterized immediately after polymerization. The semicircle in the high-frequency range (inset of Fig. 4) is indicative of the resistive and capacitive behavior of the bulk hydrogel. No semicircle is observed in the intermediary frequency range. The NLLS analysis of the data was done using an equivalent circuit without CPE_{mtd} . The determined parameters are summarized in Table 1. The results indicate that the impedimetric behavior of hydrogels immediately after polymerization is primarily capacitive. The magnitude of CPE_{dl} was on average lower than for hydrogels after conditioning in electrolyte and the exponent of CPE_{dl} (α) approached 1, which is similar to the behavior of a chemical modification leading to decreased double layer capacitance [34]. R_{ct} and Z_{wo} could not be determined at all. Fig. S6 shows a Bode plot presentation (impedance

magnitude ($\log_{10}$ scale) and phase angle vs. frequency on a $\log_{10}$ scale) of the same impedance spectrum as the one in Fig. 4. The Bode plot presentation confirms the capacitive behavior in the high-frequency range (from 1 MHz to 100 kHz) as well as the lack of a clearly defined R_{ct} and Z_{wo} . The fact that CPE_{mtd} was not required for curve fitting may be explained by the fact that the covalently tethered hydrogel domain was structurally similar to the bulk when hydration was not complete. However, the resistance of the bulk hydrogel ($R_{par,hg}$) was comparable to the one obtained after conditioning.

Fig. 4

3.2 Characterization of conditioned hydrogels – effect of cross-linking

Usually, higher cross-linking ratio in a hydrogel creates a more compact structure, i.e. lower swelling capacity. However, hydrophilic high-molecular weight cross-linkers, such as PEG, may introduce an opposite effect, increasing the swelling capacity due to the longer cross-links that make the polymer network more flexible [7]. To provide a clear comparison with the properties of the standard hydrogel described in section 3.1, we characterized polymers having a cross-linking ratio that was one half of (1:400) and twice as high (1:100) as the standard hydrogel.

Fig. 5A shows a characteristic Nyquist plot acquired for a hydrogel with cross-linking ratio 1:400. The inset shows a magnification of the high-frequency range. As in the case of the standard hydrogel, two semicircles can be seen, one in the high-frequency range (capacitive and resistive properties of the hydrogel) and another weakly defined one at the intermediate frequencies (R_{ct} and double layer capacitance). A cryo-SEM image of the same hydrogel is shown in Fig. 5B. The shown curve fitting was based on an equivalent circuit without CPE_{mtd} (summary in Table 1). Generally, the observed ranges of parameters based on characterization

of several hydrogels were clearly narrower than in the case of the standard hydrogel. This can be attributed to the uniformity of the microscopic features and compactness of the formed hydrogels as seen in Fig. 5B.

Fig. 5

The seen compactness and structural uniformity can also plausibly explain the fact that CPE_{mtd} was not needed. The compact bulk and covalently tethered domain may be structurally comparable. This entails that due to the decreased fraction of the long-chained highly hydrophilic PEG-DMA cross-linker the HEMA monomers determine the resulting structure, making the hydrogel more uniform and compact. Consequently, it leads to more limited (increased W_O impedance) but uniform (no CPE_{mtd}) mass transfer in the bulk hydrogel to the electrode-hydrogel interface. R_{ct} increased significantly in comparison with the standard hydrogel. A major contribution to this increase in R_{ct} may be attributed to hydrogel tethering on the electrode surface, which decreased the PEG-DMA fraction and concomitantly increased the density of covalently immobilized HEMA monomers on the acrylate functionalized electrode. This, on the other hand, decreased the active electrode area [34], which is inversely proportional to R_{ct} .

Fig. 6A shows a characteristic Nyquist plot for a hydrogel with cross-linking ratio 1:100. As shown in the inset, even in this case a second semicircle, albeit only partially formed, can be seen in the high-frequency range. The shown NLLS analysis required the equivalent circuit shown in Scheme 1 including CPE_{mtd} (summarized in Table 1). The fact that the analysis of 1:100 and 1:200 cross-linked hydrogels required CPE_{mtd} , whereas in the case of 1:400 cross-linked hydrogels it was not necessary, leads to the conclusion that the covalent tethering on the microchip surface causes more significant mass transfer difference when the permeability of

the bulk hydrogel increases. In comparison with the standard hydrogel, the impedimetric behavior indicates increased mass transfer to the electrode-hydrogel interface (decreased Z_{wo} impedance), decreased bulk resistance ($R_{par,hg}$), and decreased R_{ct} (Table 1). This is in accordance with the expected behavior due to the increased fraction of the high-molecular weight cross-linker. A cryo-SEM image of the same hydrogel is shown in Fig. 6B. Considering the size distribution of the pores, the impedimetric behavior is apparently contradictory. The pore dimensions are smaller than in the less cross-linked hydrogels while the mass transfer is increased. On the other hand, comparison between this hydrogel and the one in Fig. 5B points out one significant difference aside from the determined pore size distributions. The pores of the hydrogel in Fig. 6B appear to be deeper than the ones in Fig 5B, which may be an indication of greater interconnectivity of the pores throughout the bulk hydrogel.

Fig. 6

Although cryo-SEM does not show the structure of hydrated hydrogels, it provides a qualitative understanding of the structural differences [33]. To confirm the presented findings, pore interconnectivity in cross-linked hydrogels could be studied using a two-chamber measurement setup with a hydrogel partition [32,33] or the EIS method developed by Canali et al. [49], which rely on the effect of porosity on the membrane resistance or the apparent solution resistance.

The degree of hydration (calculated as described in section 2.6) of hydrogels with 1:100, 1:200 and 1:400 molar ratio of PEG-DMA and HEMA monomers was $50 \pm 1 \%$, $49 \pm 2 \%$ and $50 \pm 6 \%$ (average $\pm$ standard deviation, $n = 4$), respectively, after 24-h hydration that was comparable to the conditioning performed prior to EIS measurements. Based on two-tailed student's t-test, the degree of hydration of the hydrogels was not significantly different (5 % level; details in the Supplementary Material S7). Considering this result and the impedimetric

behavior of the differently cross-linked covalently tethered hydrogels, as presented above, it is clear that although the varying cross-linking ratio does not significantly change the swelling capacity, it drastically influences mass transfer, active electrode area, and electrode performance due to changes in the micro- and nanoenvironment at the electrode-hydrogel interface. Further studies on the state of bound water in hydrated hydrogels using, for instance differential scanning calorimetry [33,50], may provide possibility for more detailed correlation with EIS characterization.

4. Conclusion

To provide a deeper understanding of how cross-linking and covalent tethering on an electrode chip influences the impedimetric behavior of thick hydrogels in a wide frequency range, we performed a thorough EIS characterization of biocompatible hydrogels composed of poly(2-hydroxyethyl methacrylate) (pHEMA) cross-linked with hydrophilic high molecular weight poly(ethylene glycol)dimethacrylate (PEG-DMA). The obtained results demonstrated that an increased PEG-DMA/HEMA ratio (1:400 – 1:200 – 1:100) enhanced permeability. When using $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as the electroactive probe, the increased cross-linking ratio significantly decreased the charge transfer resistance due to both increased mass transfer and less dense tethering on the electrode surface modified with methacrylate moieties. Equivalent circuit modeling showed that covalently tethered thick hydrogels required an open-boundary finite-length Warburg element (Z_{wo}) to describe the mass transfer behavior. Increased cross-linking ratio was also shown to cause a difference in mass transfer at the electrode-hydrogel interface in comparison with the bulk hydrogel, requiring an additional constant phase element in series with Z_{wo} to describe the mass transfer deviation in the lowest frequency range. Cryo-SEM imaging indicated that the formed porosity was highly dependent on the cross-linking ratio and

provided a good correlation with the EIS results. On the other hand, degree of hydration of the differently cross-linked hydrogels did not show any statistically significant differences. The presented study and the proposed equivalent circuit model provide a possibility for fast data analysis in applications relying on hydrogel modified electrodes. The results can serve as a basis for further studies of diverse functionalized hydrogel systems in on-chip applications using, for instance, membrane protein carrying lipid bilayers.

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Table 1. Typical ranges for the EIS parameters obtained based on NLLS fit of impedance spectra acquired for several 1 mm thick HEMA hydrogels cross-linked with PEG-DMA in ratios 1:100, 1:200, and 1:400. (Details of the used EIS parameters can be found in the text and Supplementary Material S4.)

EIS parameters	Cross-linking ratio - (PEG-DMA:HEMA)			
	After polymerization	24 h after conditioning in PBS containing electro-active probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$		
		1:200 ^(a)	1:400 ^(a)	1:100 ^(a)
R_s/Ω ^(b)	225	225	225	225
Q_{dl}/nSs^α ^(c)	180 - 270	270 - 475	235 - 280	340 - 1125
α_{dl} ^(c)	0.920 - 0.945	0.820 - 0.920	0.880 - 0.910	0.740 - 0.820
$R_{ct}/k\Omega$	nd ^e	85 - 105	435 - 470	0.9 - 16
$Y_{wo}/\mu Ss^{1/2}$ ^(d)	nd ^e	8.3 - 11.6	0.9 - 2	16 - 49
$B_{wo}/s^{1/2}$ ^(d)	nd ^e	0.03 - 0.2	0.9 - 1.6	0.06 - 2.4
$R_{par,hg}/k\Omega$	3.4 - 3.5	3.5 - 5.8	1 - 1.7	0.20 - 0.23
$C_{par,hg}/pF$	520 - 535	430 - 500	580 - 600	720 - 880
$Q_{mtd}/\mu Ss^\alpha$ ^(c)	n/a ^f	2.9 - 11	n/a ^f	245 - 430
α_{mtd} ^(c)	n/a ^f	0.66 - 0.68	n/a ^f	0.31 - 0.54

^(a) Equivalent circuits for NLLS analysis: conditioned 1:200 and 1:100 cross-linked hydrogels (Scheme 1); 1:200 cross-linked hydrogels after polymerization and 1:400 cross-linked conditioned hydrogels (the same as in Scheme 1 without CPE_{mtd})

^(b) The average value of R_s (also comprising possible contact resistance) obtained in several tests

^(c) Q – magnitude of CPE impedance expressed as admittance (Supplementary Material S4); α – exponent acquiring values between 0 and 1 (Q_{dl} and Q_{mtd} correspond to CPE_{dl} and CPE_{mtd} in the equivalent circuit model of Scheme 1)

^(d) Y_{wo} – magnitude of Z_{wo} impedance expressed as admittance (Supplementary Material S4); B_{wo} – characteristic time constant

^(e) nd - not defined during NLLS regression analysis

^(f) n/a - not applicable due to the choice of equivalent circuit model

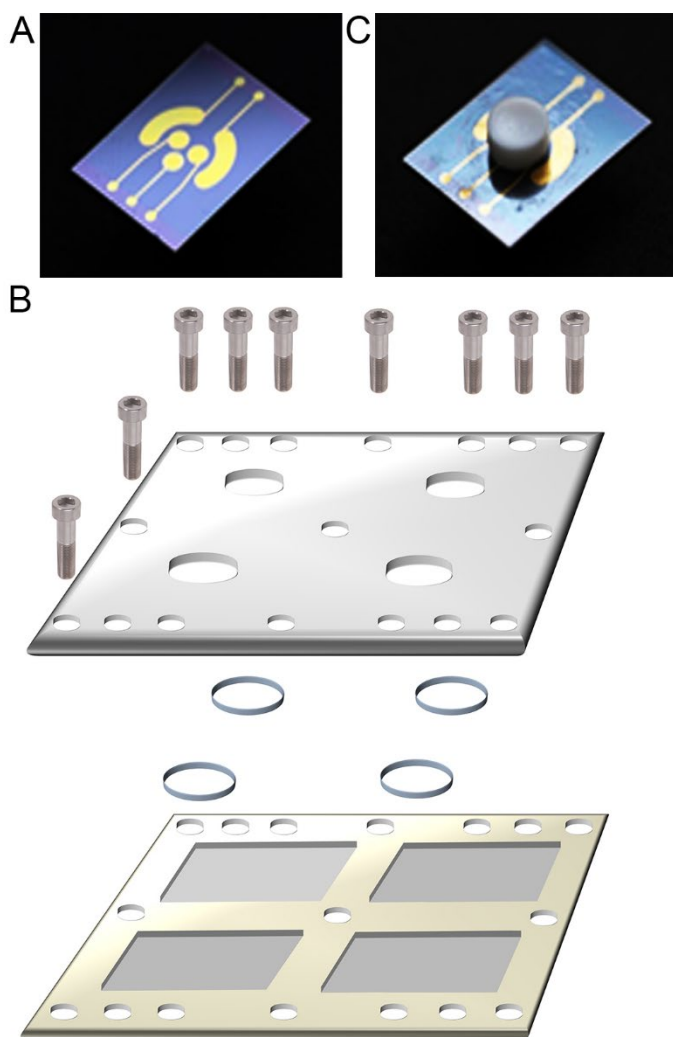


Fig. 1. Electrode microchip and hydrogel polymerization setup: A) A photo of a gold electrode microchip comprising three circular working electrodes (WE, Ø 1.5 mm) placed in the center, and two larger electrodes used as counter (CE) and reference electrode (RE) during electrochemical characterization. The electrode structures, leads and contact pads were defined using a UV lithographic process followed by reactive ion etching to define the active electrode and contact pad areas in the 500 nm thick silicon nitride layer deposited through the plasma enhanced chemical vapor deposition. B) A schematic view of the Teflon mold used for hydrogel polymerization: a bottom plate with four recessions for placement of the electrode chips; an upper plate with four openings (Ø 6 mm; sealed using polydimethylsiloxane (PDMS) O-rings, blue) that define the structure of the polymerized hydrogels. The plates were assembled together using screws. C) A photo of an *in situ* polymerized covalently tethered hydrogel disk (thickness: 1 mm, Ø 6 mm) on a gold electrode microchip. The hydrogel covered the three WEs and the surrounding silicon nitride passivation layer.

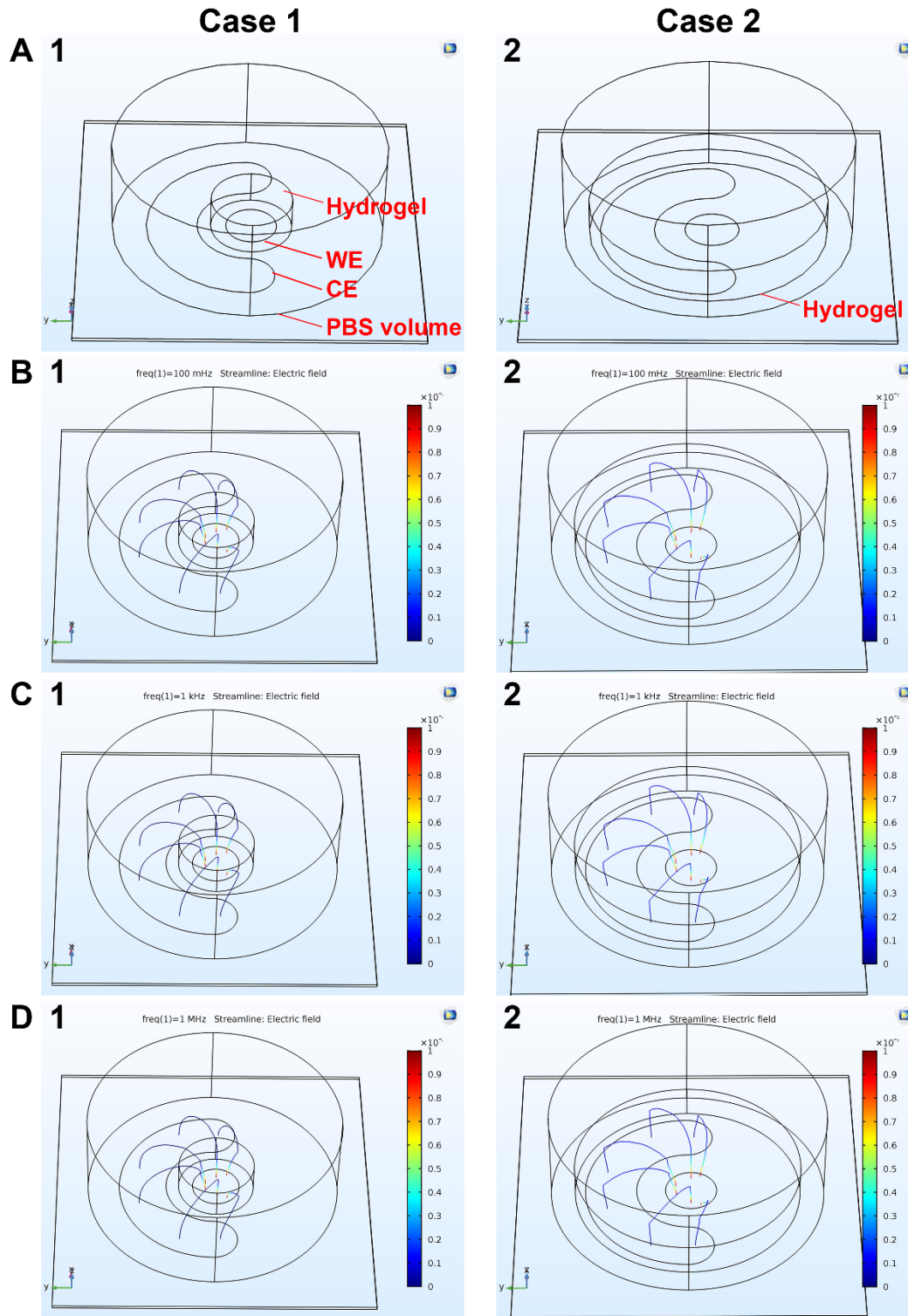


Fig. 2. Finite element simulations using COMSOL Multiphysics: A) Schematic view of the used models (case 1 – hydrogel covers only the WE; case 2 – hydrogel covers both the WE and CE). Electric field streamline propagation between the WE and CE for B) 100 mHz, C) 1 kHz, and D) 1 MHz.

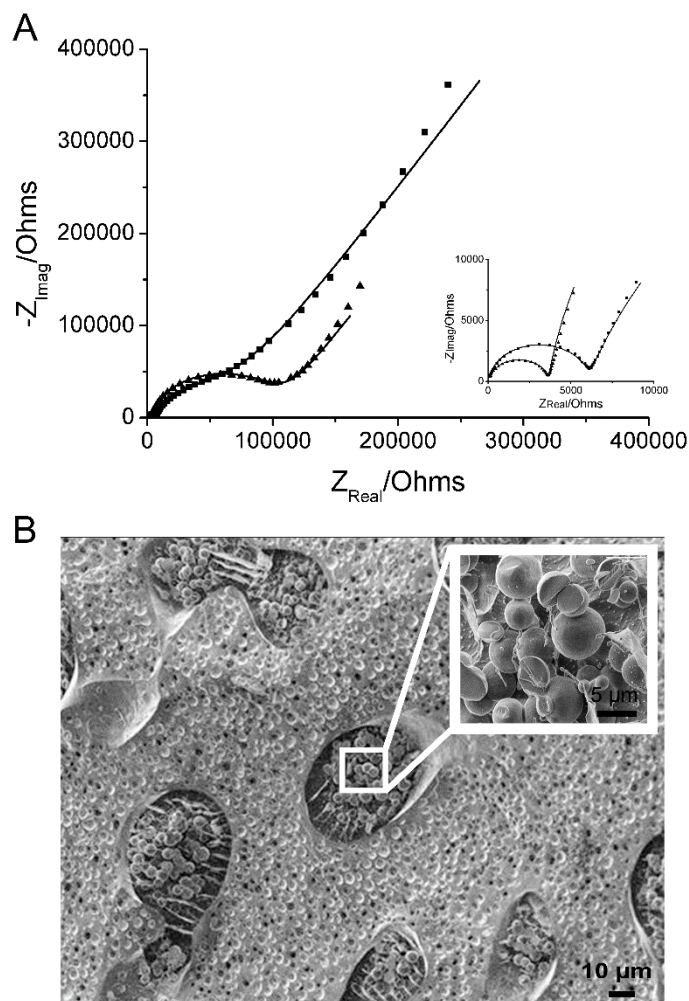
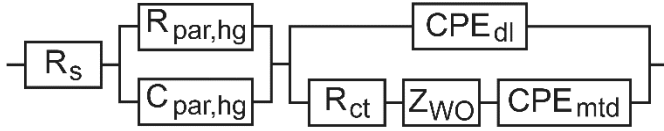


Fig. 3. Characterization of 1:200 cross-linked hydrogels after conditioning: A) Two characteristic Nyquist plots (24-h conditioning in PBS containing 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$). The EIS analysis was performed using the same electrolyte solution. The inset shows a magnified view of the high-frequency range of the Nyquist plots. The solid lines represent NLLS fit of the data to the equivalent circuit model in Scheme 1. B) A characteristic cryo-SEM image. The hydrogel forms a compact porous matrix (round pores, 2 – 3 μm in diameter) engulfing large irregular pores (dimensions from less than 30 μm to almost 200 μm). The inset shows a magnified view of the interior of a large pore.



Scheme 1. Equivalent circuit model: R_s – solution resistance, CPE_{dl} – constant phase element (accounting for the double layer capacitance and electrode surface inhomogeneity), R_{ct} – charge transfer resistance, Z_{WO} – open boundary finite-length Warburg impedance, CPE_{mtd} – constant phase element (accounting for mass transfer deviation at low frequencies), $R_{par,hg}$ and $C_{par,hg}$ – the resistance and capacitance of the bulk hydrogel, respectively.

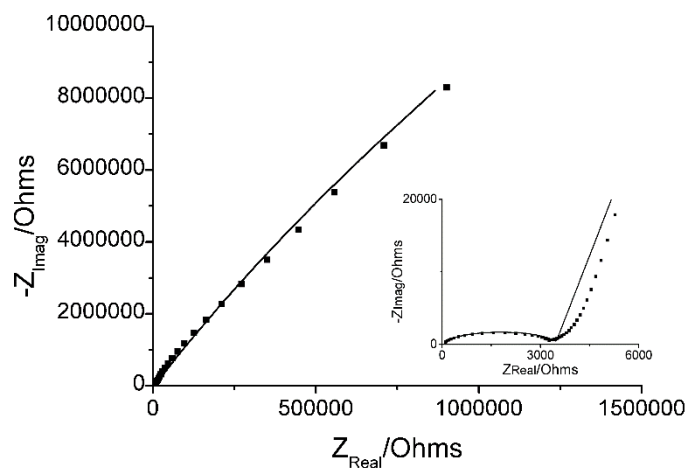


Fig. 4. Characterization of 1:200 cross-linked hydrogel immediately after polymerization: A characteristic Nyquist plot. The EIS analysis was performed using PBS containing 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$. The inset shows a magnified view of the high-frequency range of the Nyquist plot. The solid line represents NLLS fit of the data. The used equivalent circuit model was otherwise the same as in Scheme 1 except without CPE_{mtd} .

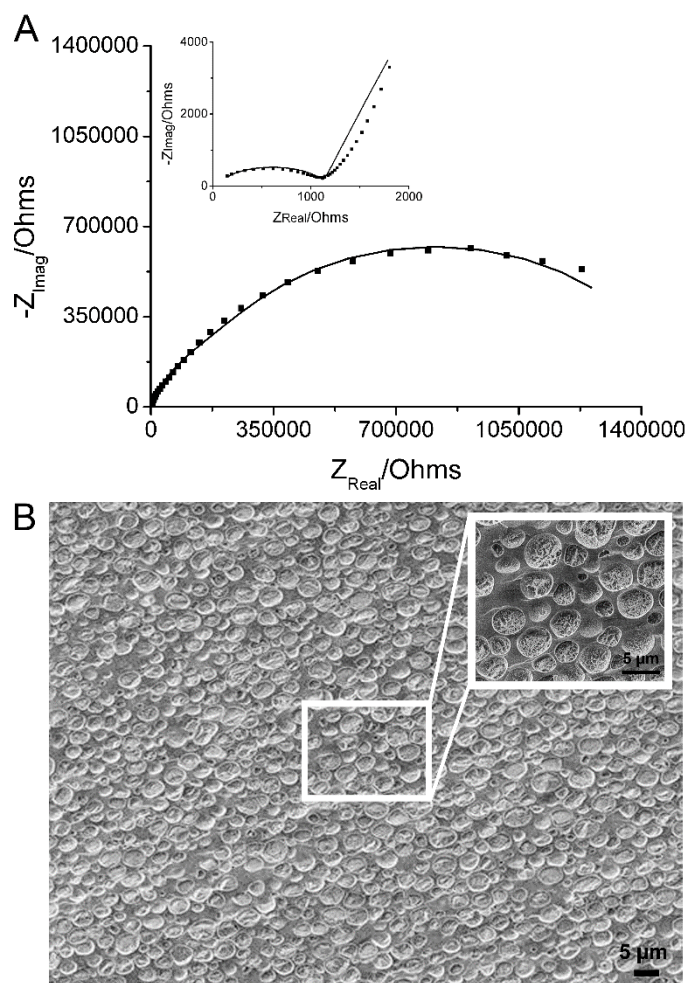


Fig. 5. Characterization of 1:400 cross-linked hydrogel after conditioning: A characteristic Nyquist plot (24-h conditioning in PBS containing 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$). The EIS analysis was performed using the same electrolyte solution. The inset shows a magnified view of the high-frequency range of the Nyquist plot. The solid line represents NLLS fit of the data. The equivalent circuit model was otherwise the same as in Scheme 1 except without CPE_{mtd} . B) A cryo-SEM image of the hydrogel. The inset shows a magnified view of the uniformly spread oval-shaped pores (minor axis: 1.5 – 3.5 μm ; major axis: 2 – 4.5 μm).

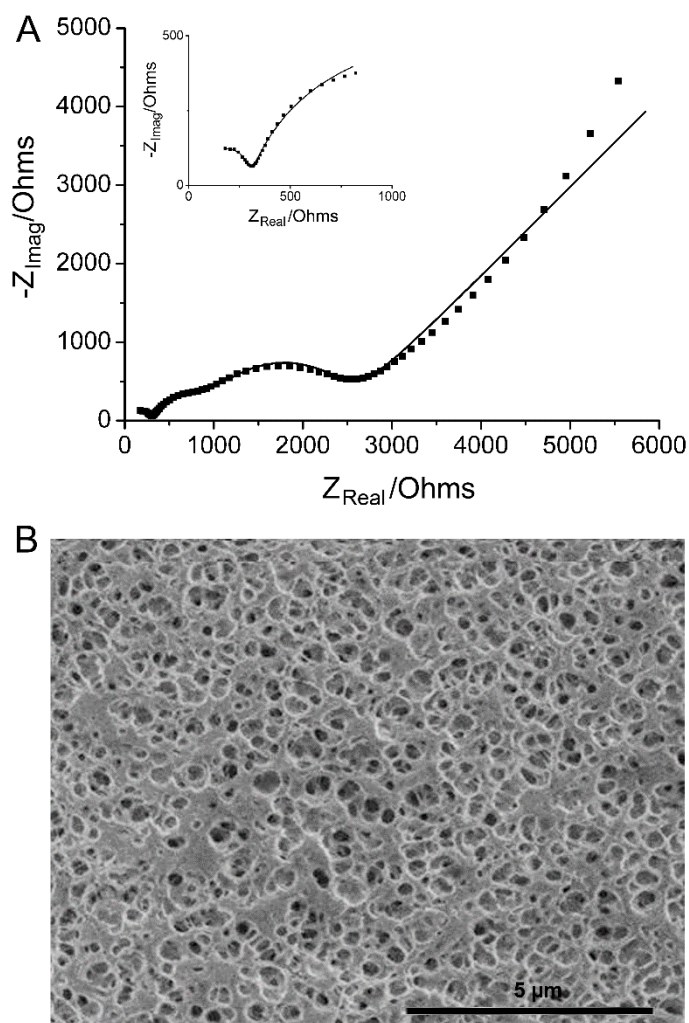


Fig. 6. Characterization of 1:100 cross-linked hydrogel after conditioning: A characteristic Nyquist plot (24-h conditioning in PBS containing 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$). The EIS analysis was performed using the same electrolyte solution. The inset shows a magnified view of the high-frequency range of the Nyquist plot. The solid line represents NLLS fit of the data to equivalent circuit model in Scheme 1. B) A cryo-SEM image of the hydrogel. The pores are nearly circular (diameters: 200 – 500 nm).

SUPPLEMENTARY MATERIAL

Impedance characterization of biocompatible hydrogel suitable for biomimetic lipid membrane applications

**Agnieszka Mech-Dorosz^{1,2}, Muhammad Salman Khan¹, Ramona Valentina Mateiu³,
Claus Hélix-Nielsen^{4,5}, Jenny Emnéus¹, Arto Heiskanen^{1,*}**

¹Technical University of Denmark, Department of Biotechnology and Biomedicine, Produktionstorvet, Building 423, DK-2800 Kgs. Lyngby, Denmark

²Present address: Novo Nordisk A/S, Brennum Park 24 K, 3400 Hillerød, Denmark

³Technical University of Denmark, Center for Electron Nanoscopy, Fysikvej, Building 307, 2800 Kgs. Lyngby, Denmark

⁴Technical University of Denmark, Department of Environmental Engineering, Bygningstorvet, Building 115, DK-2800 Kgs. Lyngby, Denmark

⁵Aquaporin A/S, Nymøllevej 78, DK-2800 Kgs. Lyngby, Denmark

*Corresponding Author

Phone: +45-45-256839

e-mail: arhe@dtu.dk

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- S6 Bode plot – 1:200 cross-linked hydrogel immediately after polymerization**
- S7 Degree of hydration for cross-linked hydrogels**

S1 Electrochemical cell for characterization of electrode modifications

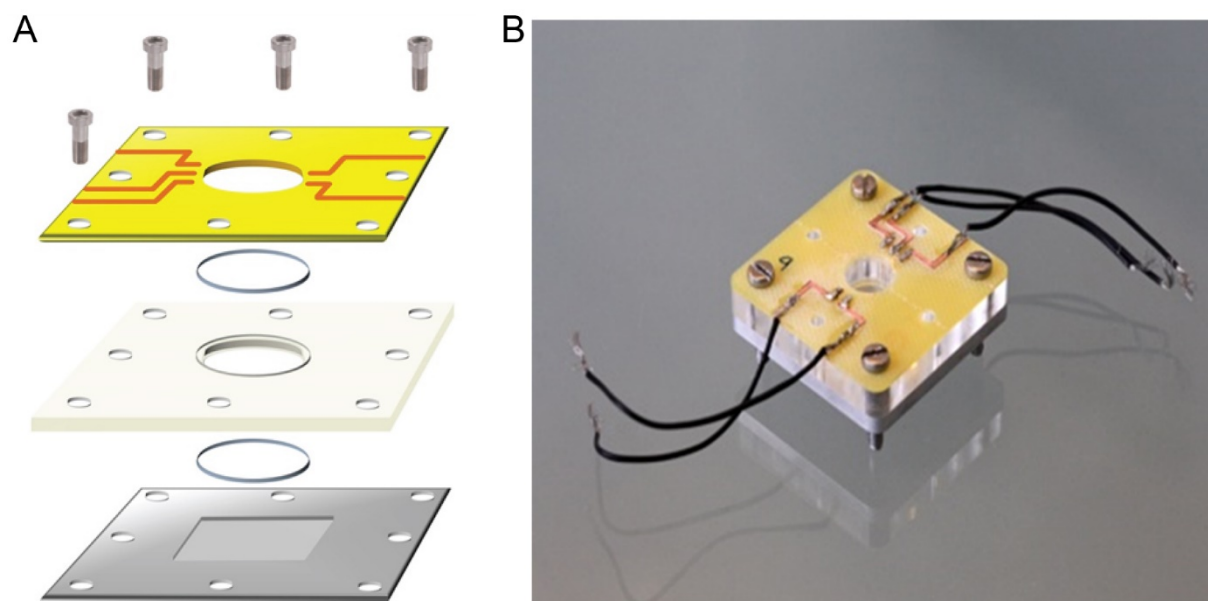


Fig. S1. Electrochemical cell for characterization of chemical electrode modifications.

A) The system comprises two micromilled plates: 1) the bottom metal plate (grey) with a recession for electrode microchip placement and 2) poly(methyl methacrylate) (PMMA) plate (white) which defines the electrochemical cell (height 5 mm), and a micromilled printed circuit board (PCB, yellow) with spring loaded pins for electric connections to the inserted electrode microchip. The diameter of the electrochemical cells is 10 mm facilitating access to the three working electrodes (in the middle) and the two larger electrodes used as on-chip counter and reference electrode. Fluid tight connection between the electrode microchip and the PMMA plate is achieved by using a polydimethylsiloxane (PDMS) O-ring (blue), prepared by casting PDMS into a micromilled mold. Another PDMS O-ring was placed between the PMMA plate and PCB to avoid that the electrolyte in the electrochemical cell creeps between the PMMA plate and PCB. B) A photo of an assembled setup.

S2 EIS characterization of chemical modifications of an electrode microchip

The performed chemical modifications of electrode microchips comprised three steps aiming at formation of hydroxyl functionalities followed by silanization to introduce methacrylate moieties on both electrode surfaces (gold) and the surrounding silicon nitride passivation layer. The influence of each modification step was characterized by electrochemical impedance spectroscopy (EIS) using the on-chip counter and reference electrode (frequency range: 100 mHz – 1 MHz; 10 data points per decade; 10 mV_{rms} sinusoidal potential) in the presence of equimolar [Fe(CN)₆]^{3-/4-} (10 mM) in PBS. Figure S2 shows characteristic Nyquist plots acquired for one working electrode. Data were fitted to the modified Randles circuit (shown schematically in the inset of Figure S2) that represents the solution resistance in series with a parallel circuit composed of a constant phase element (CPE), accounting for double layer capacitance and surface inhomogeneity, parallel with charge transfer resistance (R_{ct}) and Warburg impedance (W). Hydroxylation of silicon nitride surface using H₂O₂ strongly oxidizes gold, increasing the charge transfer resistance. A further increase in R_{ct} was observed after β -mercaptoethanol SAM formation on gold (introduces hydroxyl functionalities for silanization) and silanization by 3-(trimethoxysilyl propyl) methacrylate of both the nitride and gold surfaces, indicating formation of a “physical barrier” for electroactive species. The oxidation of the gold surface as a consequence of the hydroxylation step resulted in a significant increase in CPE corresponding to increased capacitance analogously to what was shown in our previous work using PBS without any electroactive species [1]. The further chemical modifications decreased the CPE as an indication of decreased capacitance since such capacitive contributions to the overall impedance behave as series capacitors [2]. The value of W was only negligibly influenced by the modification steps.

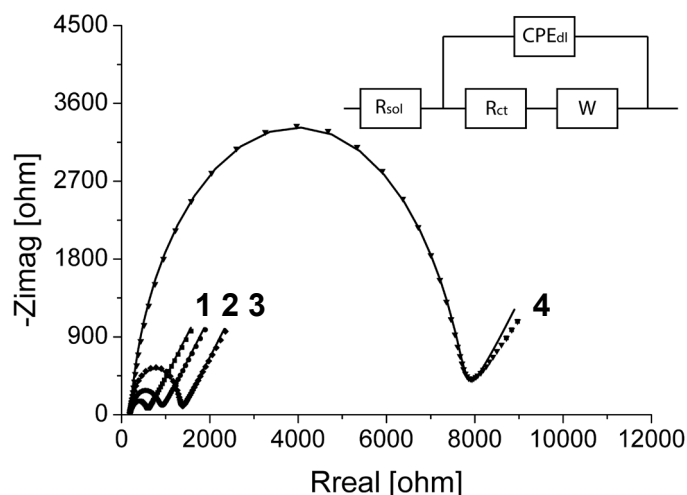


Fig. S2. Nyquist plots acquired on 1) a cleaned electrode (▲), as well as the same electrode after 2) hydroxylation (H₂O₂ treatment) of the gold electrode microchip (●), 3) β -mercaptoethanol SAM formation on the H₂O₂ treated electrode (◆), and 4) silanization of β -mercaptoethanol SAM with 3-(trimethoxysilyl propyl) methacrylate (▼). Solid lines represent the nonlinear least square fitting of the data to the Randles equivalent circuit model (including a constant phase element, CPE_{dl} , to describe the double layer capacitance and surface inhomogeneity of the electrode) shown in the inset.

S3 Finite element simulations

A hydrogel scaffold placed on an electrode surface develops corrugated voids due to the random scaffold structure. These voids promote currents leakage, such that electric fields avoid the scaffold structure and bypass through the electrolyte, which is not desirable when characterizing hydrogel scaffolds. Tully-Dartez et al. has described this anomaly while performing a similar study between chitosan scaffolds with embedded cylindrical electrodes [3]. In our study, the electric field, originating from the applied sinusoidal potential across the complex 3D hydrogel scaffold, which was covalently tethered on the electrode surface and the surrounding silicon nitride passivation layer, must essentially penetrate through the hydrogel during the entire frequency range.

3D space dimension model along with frequency domain and time dependent study was selected, and model was constructed as shown in Figure S3-1A and Figure S3-2A, for Case 1 and Case 2, respectively. The AC/DC physics interface was selected to model electric field lines at various frequencies for both cases. Figure S3-1B-G shows the simulation results for Case 1 at the frequencies of 100 mHz, 10 Hz, 1 kHz, 10 kHz, 100 kHz and 1 MHz as side (left panels) and top (right panels) view, respectively. Similarly, Figure S3-2B-G shows the results at the abovementioned frequencies as side (left panels) and top (right panels) view for Case 2. The conductivity of PBS and pHEMA were defined based on previously published values as 1.6 S/m [4] and 3.5 mS/m [5], respectively. The mesh consisted of tetrahedral elements. The characteristic values of the mesh quantity and quality are shown in Table S3 below.

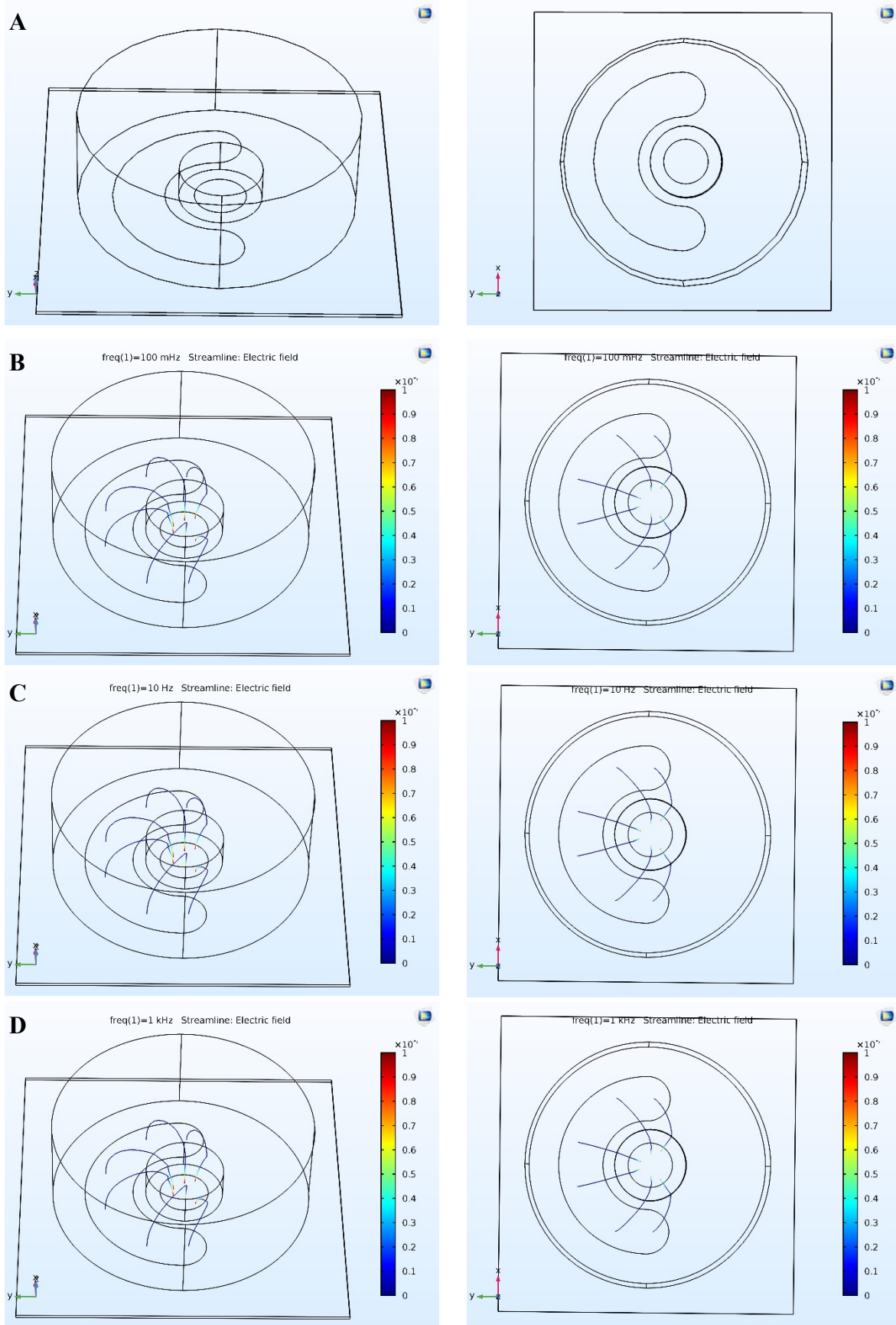
Table S3. Mesh quantity of each element and mesh quality for both cases.

Elements	Case1	Case2
Domain elements	109519	187687
Boundary elements	26402	38952
Edge elements	1491	1895
Mesh quality	0.91	0.92

Case 1 – Hydrogel covers only the WE

Side view

Top view



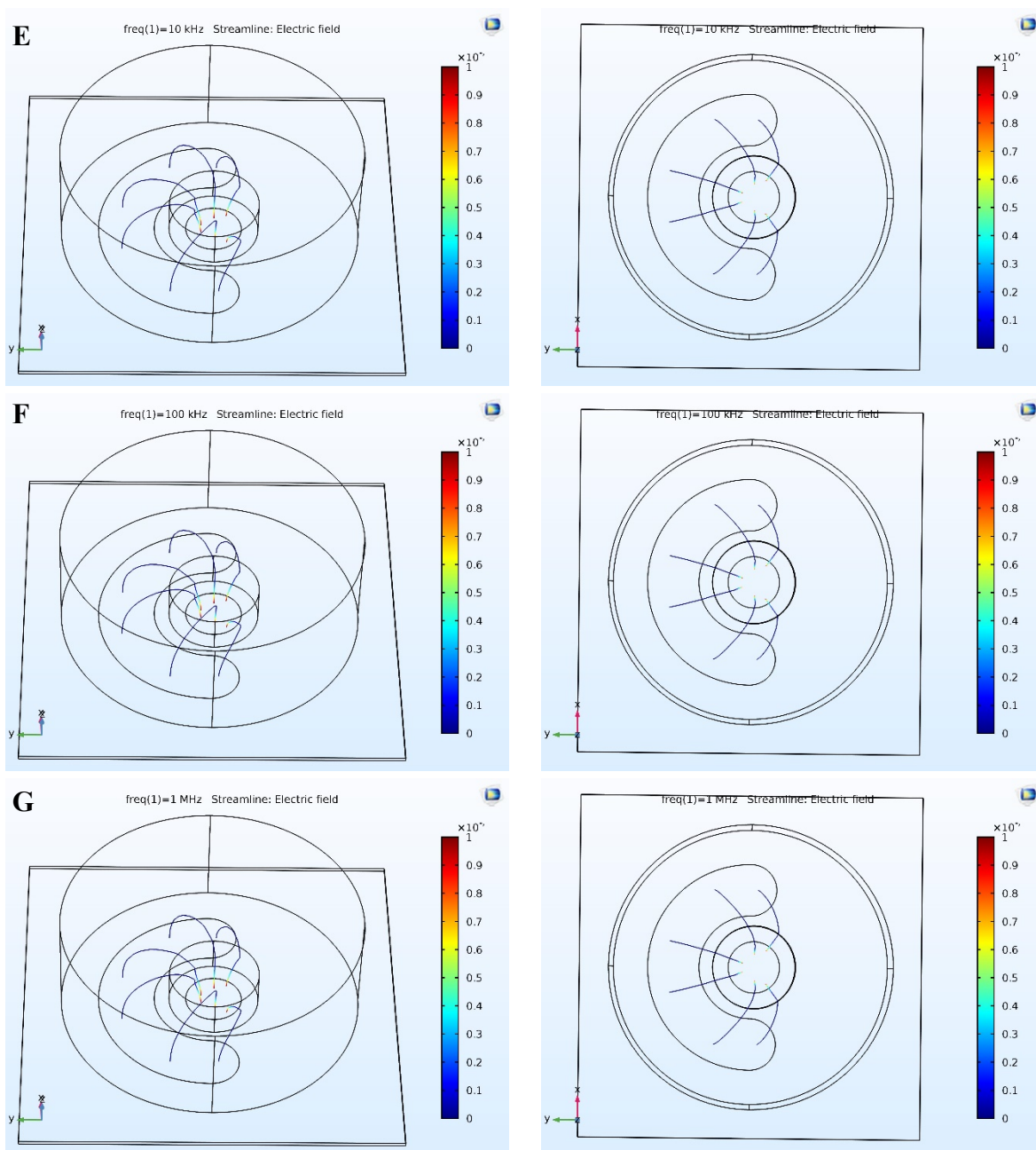
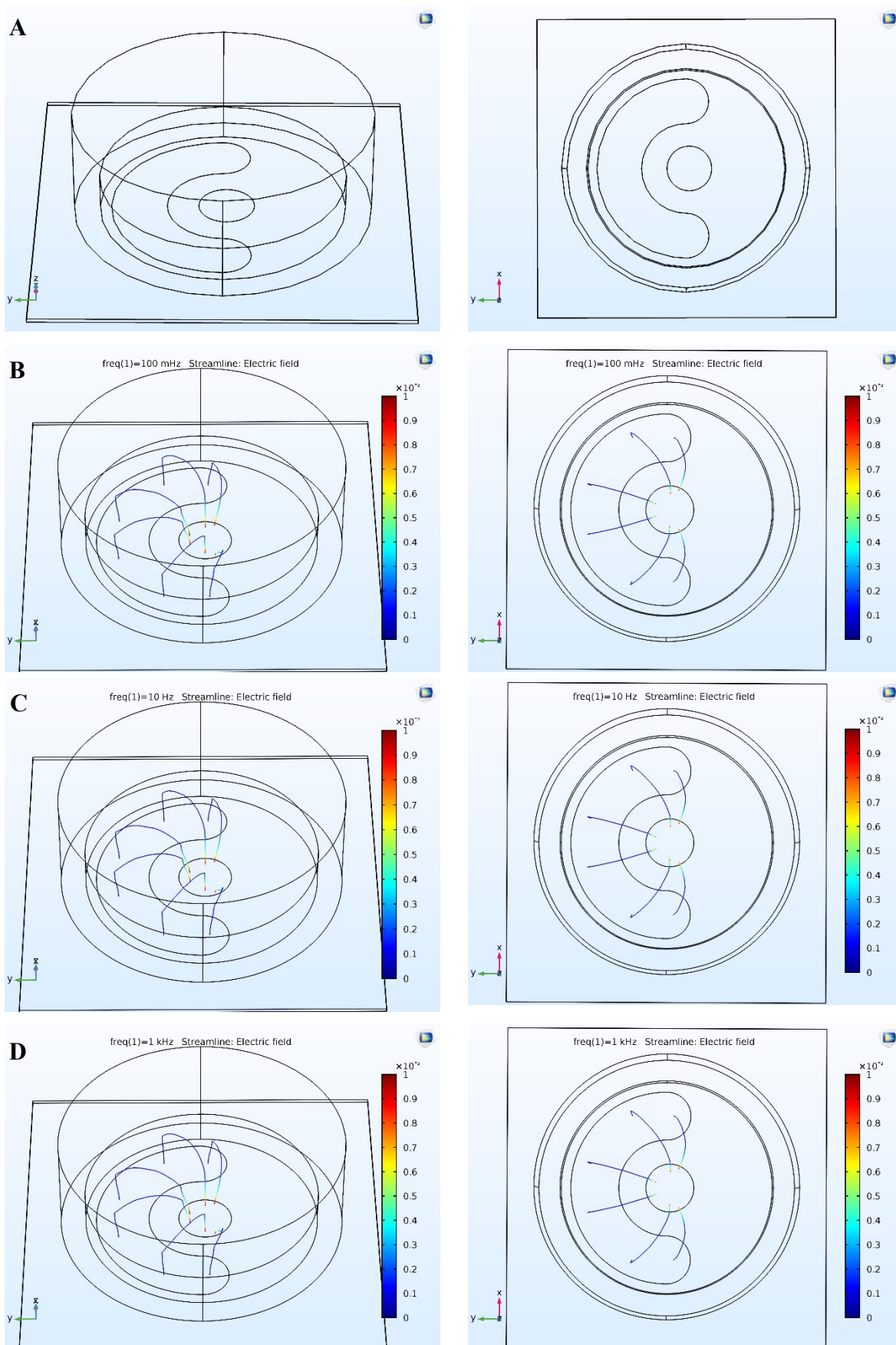


Fig. S3-1. Finite element simulations using COMSOL Multiphysics for case 1:
A) Schematic view of the used model (Left column – side view; right column – top view).
Electric field streamline propagation between the WE and CE for B) 100 mHz; C) 10 Hz; D) 1 kHz; E) 10 kHz; F) 100 kHz; G) 1 MHz.

Case 2 – Hydrogel covers both the WE and CE

Side view

Top view



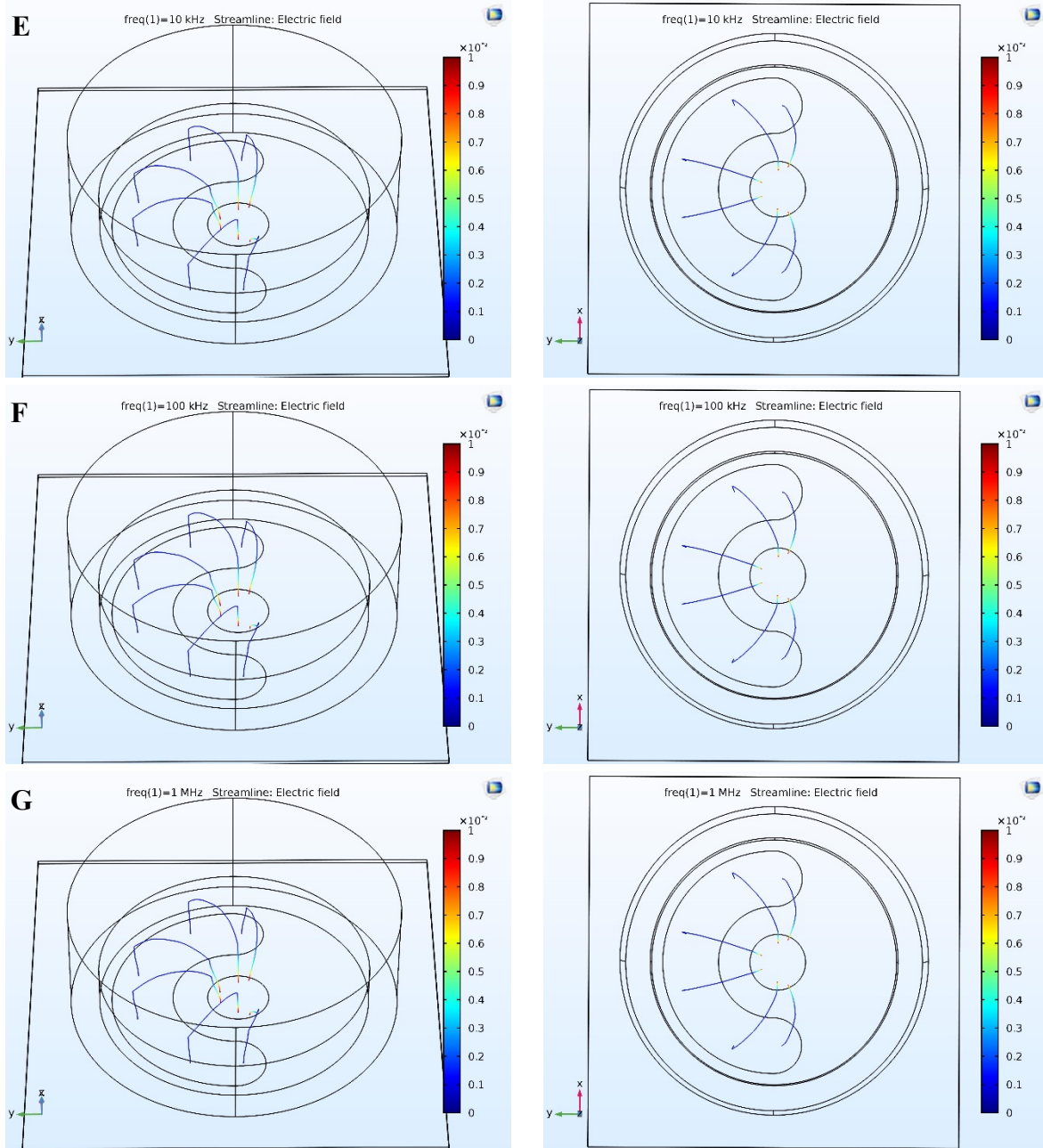


Fig. S3-2. Finite element simulations using COMSOL Multiphysics for case 2:
A) Schematic view of the used model (Left column – side view; right column – top view).
Electric field streamline propagation between the WE and CE for B) 100 mHz; C) 10 Hz; D) 1 kHz; E) 10 kHz; F) 100 kHz; G) 1 MHz.

S4 Description of impedance elements

Constant phase element (CPE)

Constant phase element (CPE) has been used to characterize the interface behavior of solid electrodes when the behavior is not purely capacitive (i.e. due to phenomena that could be attributed to, e.g., surface roughness, a certain degree of dispersion is observed instead of pure double layer capacitance behavior) [6]. It has also found applications in modeling of finite diffusion layer behavior at electrodes with a porous coating [7]. Mathematically, its impedance is expressed as shown in Eq. 1

$$\vec{Z}_{CPE} = \frac{1}{Q_0(j\omega)^\alpha} \quad \text{Eq. 1}$$

where ω is the angular frequency, $j = (-1)^{1/2}$, Q_0 the magnitude of $1/Z_{CPE}$ (Y_{CPE}) at $\omega = 1$ rad/s, α obtains values between 0 and 1. When $\alpha = 1$, Z_{CPE} represents pure capacitance ($Q_0 = C$). At the opposite limit, $\alpha = 0$, Z_{CPE} represents pure resistor. The value of α determines the constant phase angle (θ_{CPE}) according to $\theta_{CPE} = -(\pi/2)\alpha$. The unit of $|Z_{CPE}|$ is Ω , and hence, since ω^α has unit $s^{-\alpha}$, Q_0 has unit $S s^\alpha$. Q_0 is the value given by many equivalent circuit modeling programs (e.g. Gamry Echem Analyst).

In the complex impedance plane (Nyquist plot), Z_{CPE} in parallel with a resistance (e.g. charge transfer resistance) generates the well-known recessive semicircle. Fig. S4-1A illustrates a Nyquist plot generated using the equivalent circuit in Fig. S4-1B (analogous to the equivalent circuit of Scheme 1 in the article with the exception that no diffusion impedance is included).

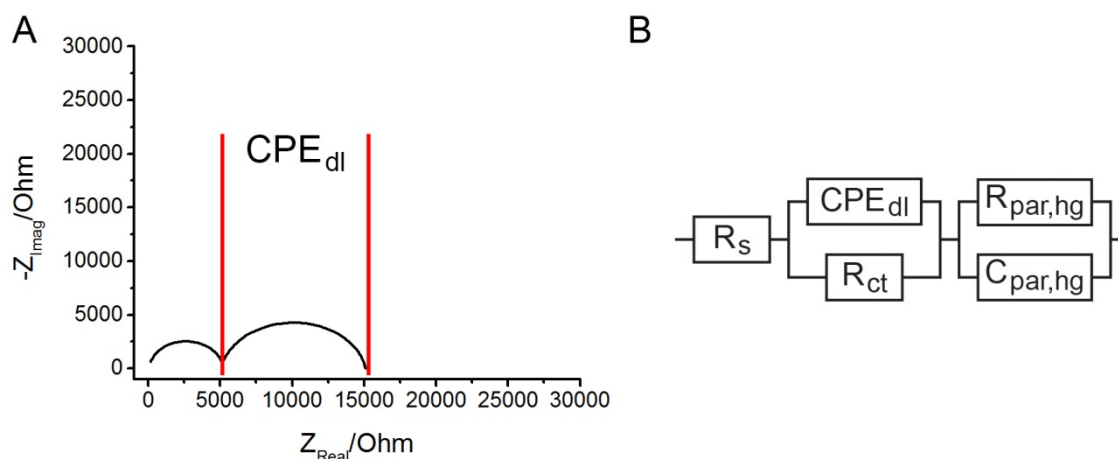


Fig. S4-1. A) Complex impedance plane representation (Nyquist plot) of impedance generated based on the equivalent circuit in B). (CPE_{dl} – Z_{CPE} related to the interface impedance comprising the double layer capacitance; other components as described in the article; generated by EIS simulation script of Gamry Echem Analyst v. 6.10 – $R_s = 100 \Omega$, $Q_{0,dl} = 500 \text{ nS s}^\alpha$ (Q_0 of CPE_{dl}), $\alpha = 0.900$, $R_{ct} = 10 \text{ k}\Omega$, $R_{par,hg} = 5 \text{ k}\Omega$, $C_{par,hg} = 800 \text{ pF}$)

Warburg impedance for semi-infinite diffusion (Z_W)

The Warburg impedance (Z_W) has been commonly used as diffusion impedance element suitable for characterizing diffusion of electroactive species to an electrode-electrolyte interface with semi-infinite thickness of the Nernst diffusion layer (δ) [8]. Mathematically, it is expressed as shown in Eq. 2 [9]

$$\vec{Z}_W(\omega) = \sigma \omega^{-1/2} - j\sigma \omega^{-1/2} \quad |Z_W| = \sqrt{2} \sigma \omega^{-1/2} \quad \text{Eq. 2a}$$

$$\sigma = \frac{RT}{n^2 F^2 A \sqrt{2}} \left(\frac{1}{D_O^{1/2} C_O^*} + \frac{1}{D_R^{1/2} C_R^*} \right) \quad \text{Eq. 2b}$$

where ω is the angular frequency, $j = (-1)^{1/2}$, R the molar gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T the absolute temperature, n the number of electrons involved, F the Faraday constant (96485 C mol^{-1}), D_O and D_R the diffusion coefficient of the oxidized and reduced species, respectively, C_O^* and C_R^* the bulk concentration of the oxidized and reduced species, respectively. The magnitude of Z_W ($|Z_W|$) has unit Ω , and hence, since $\omega^{1/2}$ has unit $\text{s}^{1/2}$, σ has unit $\Omega \text{s}^{-1/2}$. As admittance notation, $|Z_W|$ can be expressed as shown in Eq. 3

$$\frac{1}{|Z_W|} = \frac{1}{\sqrt{2} \sigma} \omega^{1/2} \quad \text{Eq. 3}$$

where $1/\sigma$ can be denoted by Y_W . Hence, using this notation $|Z_W|$ can be expressed as shown in Eq. 4

$$|Z_W| = \sqrt{2} \frac{1}{Y_W} \omega^{-1/2} \quad \text{Eq. 4}$$

Y_W having unit $\Omega^{-1} \text{s}^{1/2}$ ($\text{S s}^{1/2}$) is the value given by many equivalent circuit modeling programs (e.g. Gamry Echem Analyst). This approach facilitates an easy comparison of obtained values. The smaller the value of Y_W is the more limited is diffusion based mass transfer (i.e. the higher is $|Z_W|$).

In the complex impedance plane (Nyquist plot), Z_W generates the well-known Warburg line with 45° slope. Fig. S4-2A illustrates a Nyquist plot generated using the equivalent circuit in Fig. S4-2B (analogous to the equivalent circuit of Scheme 1 in the article with the exception that Z_W is solely representing the diffusion impedance).

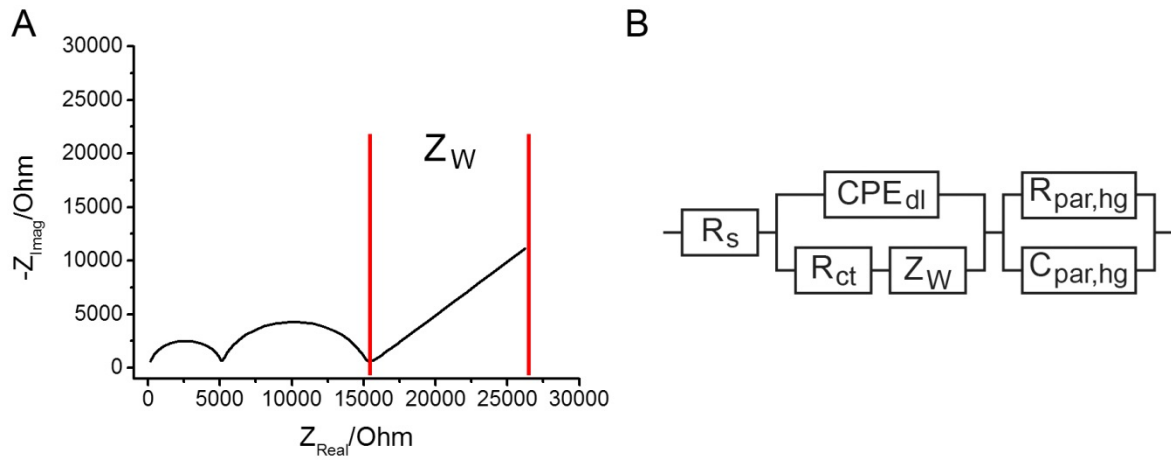


Fig. S4-2. A) Complex impedance plane representation (Nyquist plot) of impedance generated based on the equivalent circuit in B). (Z_W – semi-infinite Warburg impedance; all the other components of the equivalent circuit are the same as described in Fig. S4-1 and the article; generated by EIS simulation script of Gamry Echem Analyst v. 6.10 – $Y_W = 800 \mu S s^{1/2}$; the other components have the same values as in Fig. S4-1)

Open-boundary Warburg impedance for finite diffusion (Z_{WO})

Modeling of, for instance, porous electrode modifications with an open (unperturbed) outer boundary, leading to a finite thickness of δ , has employed open-boundary finite-length Warburg impedance (Z_{WO} , O element) [10]. Z_{WO} is a modified version of Z_W and can be expressed as shown in Eq. 5 when following the notation in Eq. 4 above.

$$\vec{Z}_{WO}(\omega) = \left(\frac{1}{Y_{WO}\sqrt{j\omega}} \right) \tanh(B\sqrt{j\omega}) \quad \text{Eq. 5a}$$

$$B = \delta/\sqrt{D} \quad \text{Eq. 5b}$$

As above, the unit of Y_{WO} is $S s^{1/2}$. The unit of B (characteristic time constant describing diffusion through δ) is $s^{1/2}$.

In the complex impedance plane (Nyquist plot), Z_{WO} generates a Warburg line with 45° slope at frequencies, $f > 2/B^2$, while at lower frequencies the curve bends toward the Z_{Real} axis. Fig. S4-3A illustrates a Nyquist plot generated using the equivalent circuit in Fig. S4-3B (analogous to the equivalent circuit of Scheme 1 in the article with the exception that Z_{WO} is solely representing the diffusion impedance).

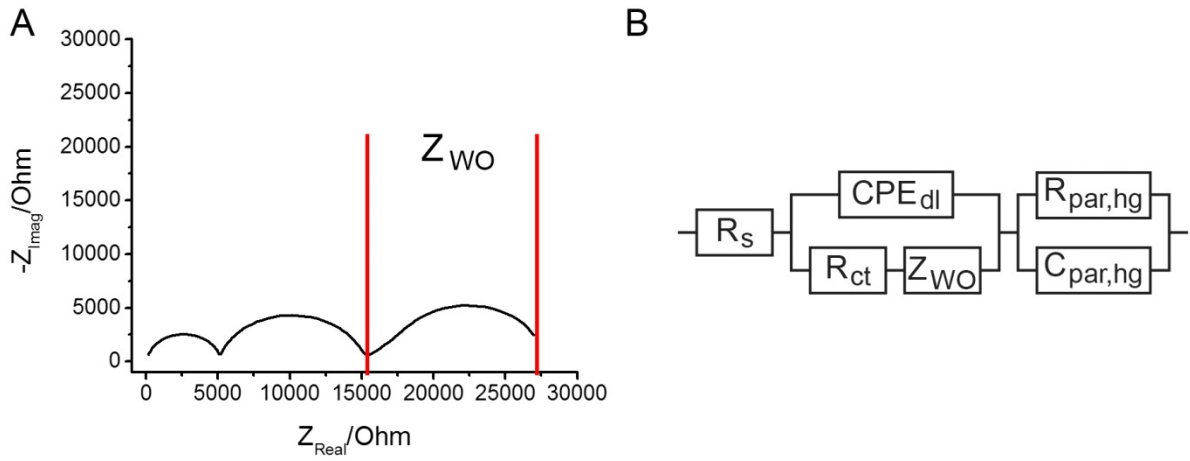


Fig. S4-3. A) Complex impedance plane representation (Nyquist plot) of impedance generated based on the equivalent circuit in B). (Z_{WO} – open-boundary finite-length Warburg impedance; all the other components of the equivalent circuit are the same as described in Fig. S4-1 and the article; generated by EIS simulation script of Gamry Echem Analyst v. 6.10 – $Y_{WO} = 800 \mu S s^{1/2}$, $B = 10 s^{1/2}$)

Blocked-boundary Warburg impedance for finite diffusion (Z_{WT})

Modeling of, for instance, porous electrode modifications with a blocked outer boundary, leading to a finite thickness of δ , has employed blocked-boundary finite-length Warburg impedance (Z_{WT} , T element) [10]. Z_{WT} is a modified version of Z_W and can be expressed as shown in Eq. 6 when following the notation in Eq. 4 analogously as in the case of Eq. 5.

$$\vec{Z}_{WO}(\omega) = \left(\frac{1}{Y_{WT}\sqrt{j\omega}} \right) \coth(B\sqrt{j\omega}) \quad \text{Eq. 6}$$

The unit of Y_{WT} is the same as for Y_{WO} . B is defined as shown above (Eq. 5b).

In the complex impedance plane (Nyquist plot), Z_{WT} generates a Warburg line with 45° slope at frequencies, $f > 2/B^2$, while at lower frequencies the curve turns into a 90° capacitive line. Fig. S4-4A illustrates a Nyquist plot generated using the equivalent circuit in Fig. S4-4B (analogous to the equivalent circuit of Scheme 1 in the article with the exception that Z_{WT} is solely representing the diffusion impedance).

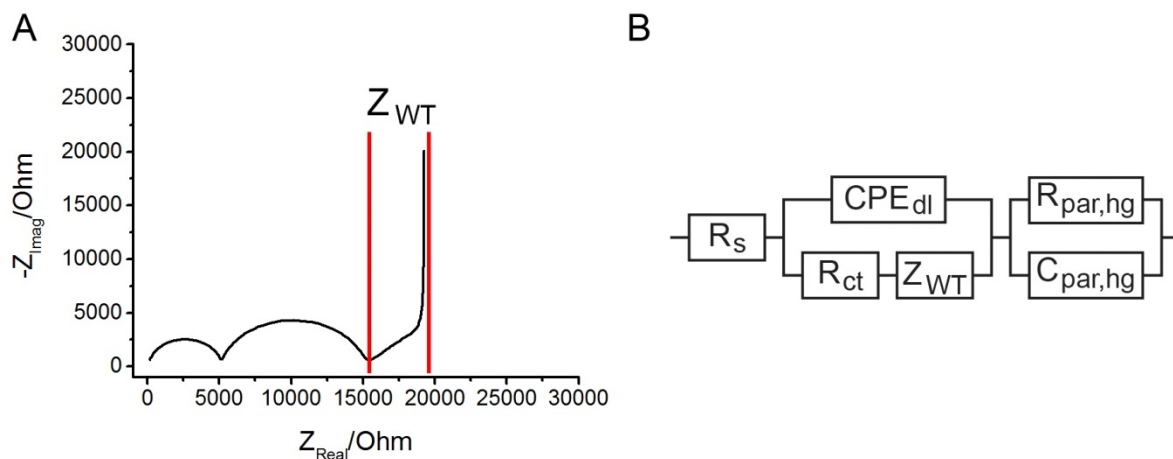


Fig. S4-4. A) Complex impedance plane representation (Nyquist plot) of impedance generated based on the equivalent circuit in B). (Z_{WT} – blocked-boundary finite-length Warburg impedance; all the other components of the equivalent circuit are the same as described in Fig. S4-1 and the article; generated by EIS simulation script of Gamry Echem Analyst v. 6.10 – $Y_{\text{WT}} = 800 \mu\text{Ss}^{1/2}$, $B = 10 \text{ s}^{1/2}$)

S5 NLLS regression analysis using alternative equivalent circuit models

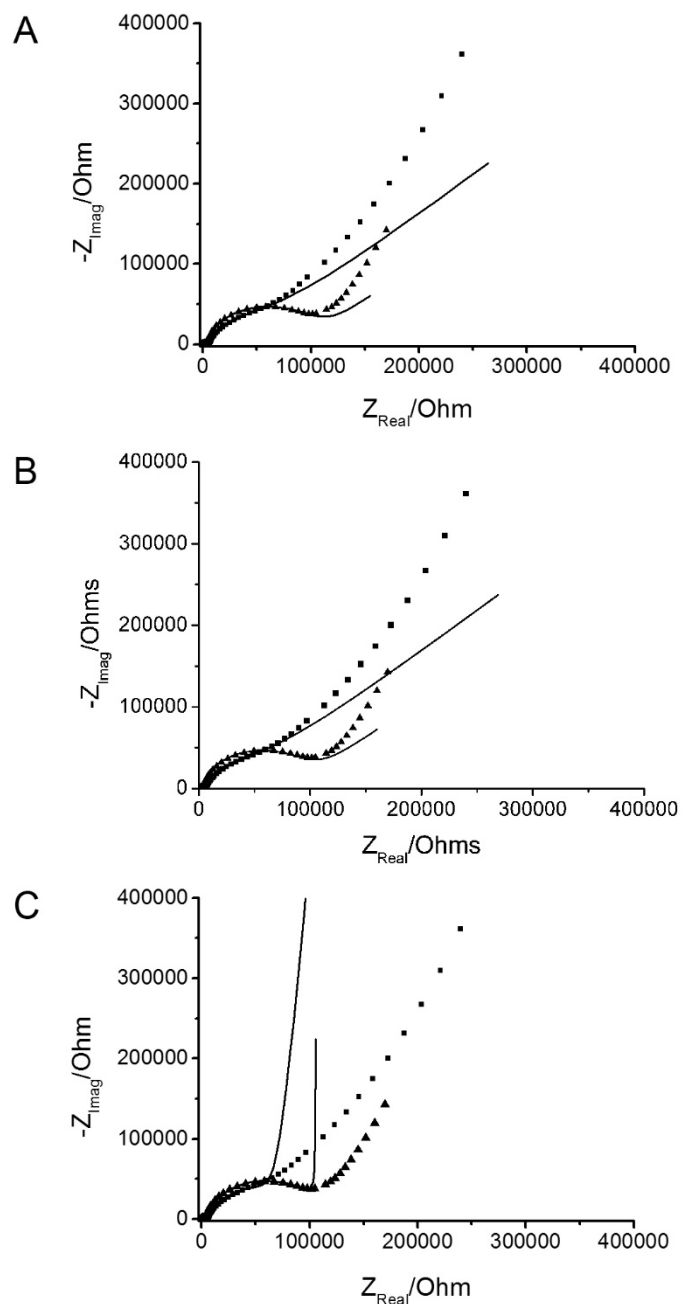


Fig. S5. NLLS regression analysis of Nyquist plots (Fig. 2A in the article) acquired for 1:200 cross-linked conditioned hydrogel using the equivalent circuit model shown in A) S4-2B, B) Scheme 1 of the article without CPE_{mtd} , and C) Scheme 1 of the article except that Z_{wo} was replaced by Z_{WT} .

S6 Bode plot – 1:200 cross-linked hydrogel immediately after polymerization

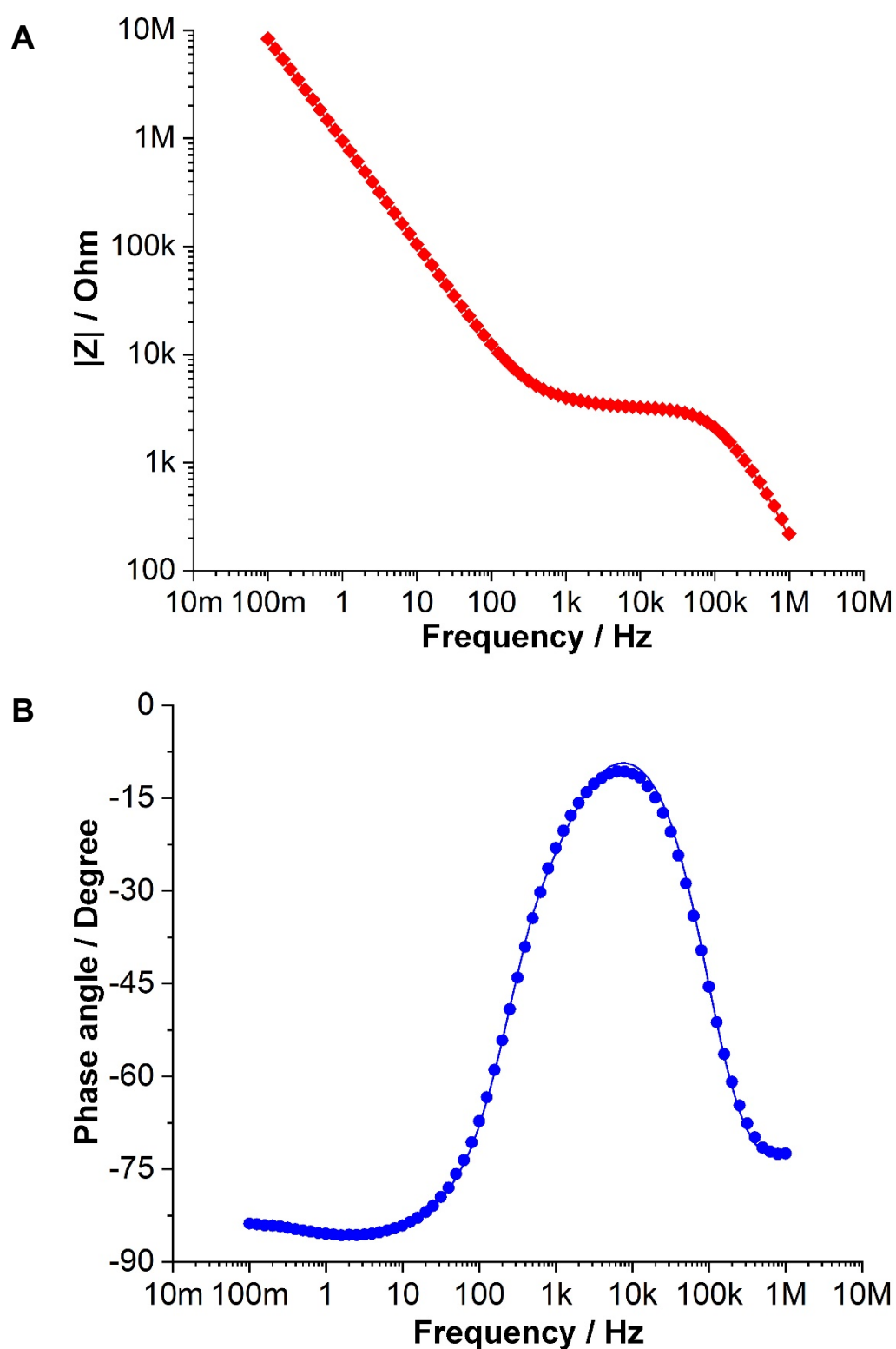


Fig. S6. Characterization of 1:200 cross-linked hydrogel immediately after polymerization: A characteristic Bode plot showing A) impedance magnitude and B) phase angle. The EIS analysis was performed using PBS containing 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$. The solid line represents NLLS fit of the data. The used equivalent circuit model was otherwise the same as in Scheme 1 except without CPE_{mtd} .

S7 Degree of hydration for cross-linked hydrogels

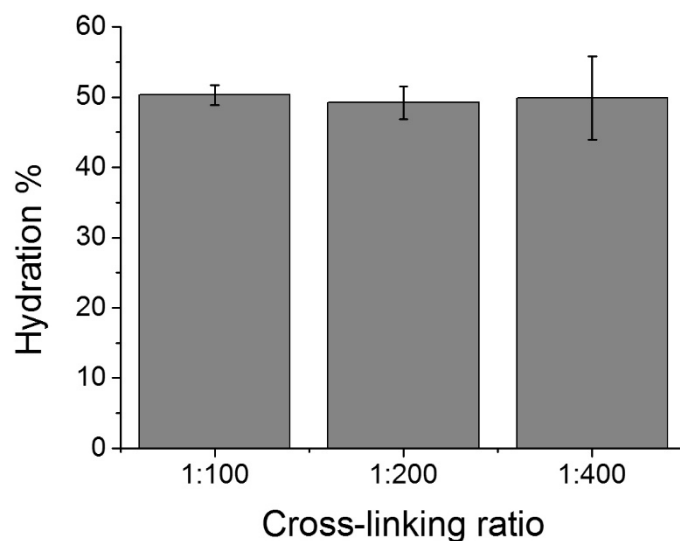


Fig. S7. Degree of hydration presented as $\text{Hydration \%} = [(w_{\text{wet}} - w_{\text{dry}})/w_{\text{wet}}] \times 100\%$ [11]; Average $\pm$ standard deviation ($n = 4$).

Table S7. Statistical analysis of hydration % (cross-linking ratio PEG-DMA¹:HEMA²)

Two-sample tests (5% level)	1:100	1:200	1:400
	Normal distribution ³ p = 0.9885	Normal distribution ³ p = 0.2376	Normal distribution ³ p = 0.4540
1:100		Equal variance ⁴ p = 0.4462 t-test ⁵ p = 0.4519	Unequal variance ⁴ p = 0.0440 t-test ⁵ p = 0.8973
1:200			Equal variance ⁴ p = 0.1613 t-test ⁵ p = 0.8379

¹ PEG-DMA – Poly(ethylene glycol)-dimethacrylate

² HEMA – 2-hydroxyethyl methacrylate

³ Normality test: Shapiro-Wilk (OriginPro 9.0)

⁴ Two-tailed F-test (OriginPro 9.0)

⁵ Two-tailed t-test (OriginPro 9.0)

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