RESEARCH ARTICLE

Macromolecular Bioscience
www.mbs-journal.de

Analysis of the Binding of Cytokines to Highly Charged Polymer Networks

*Uwe Freudenberg, Passant Atallah, Jens-Uwe Sommer, Carsten Werner, and Matthias Ballauff**

A model describing the binding of biological signaling proteins to highly charged polymer networks is presented. The networks are formed by polyelectrolyte chains for which the distance between two charges at the chain is smaller than the Bjerrum length. Counterion condensation on such highly charged chains immobilizes a part of the counterions. The Donnan-equilibrium between the polymer network and the aqueous solution with salt concentration c_s^b is used to calculate the salt concentration of the co- and counterions c_s^g entering the network. Two factors are decisive: i) The electrostatic interaction between the network and the protein is given by the Donnan-potential of the network and the net charge of the protein. In addition to this leading term, a second term describes the change in the Born-energy of the proteins when entering the network. ii) The interaction of the protein with the highly charged chains within the network is governed by counterion release: Patches of positive charge at the protein become multivalent counterions of the polyelectrolyte chains thus releasing a concomitant number of condensed counterions. The model compares favorably to experimental data obtained on a set of biohybrid polymer networks composed of crosslinked glycosaminoglycan chains that interact with a mixture of key signaling proteins.

1. Introduction

Polymer networks bearing charges have been a long-standing subject of polymer science.[1,2] Immersed in water these hydrogels will swell until the osmotic pressure of the counterions inside the gel is balanced by the retractive force of the polymer networks.[1] The salt concentration within the gel is adjusted by the Donnan-equilibrium with the reservoir and the degree of swelling of the network is depending on the ionic strength. These basic features are largely understood by now[1,2] and the analytical theory of weakly charged networks, that is, of networks where the distance between the charges along the polymer chain b is more than the Bjerrum length l_B is well-developed.[3,4]

Charged hydrogels can take up and bind proteins from aqueous solution. This phenomenon has been discussed by various authors and a review can be found in ref.[5] Charged microgels are particularly suited to study this process since adsorption equilibrium can be attained in a short time.[6–12] This fact could be used to investigate the dependence of protein adsorption on various parameters, such as, e.g., the ionic strength in the system.[13] Moreover, adsorption of two different proteins has been studied[14] which presents an important step towards a quantitative investigation of the competitive adsorption and desorption of a mixture of proteins with temporal resolution (Vroman effect; see the discussion in ref.[15]). Micron-sized gel particles are equally well-suited to analyze and model the kinetics of protein uptake with spatial resolution.[9] These investigations have clearly revealed that the diffusion of the proteins to the surface of the gel is the rate-determining step.[9,10]

A quantitative model for the uptake of proteins to weakly charged networks has been developed some time ago by Yigit, Dzubiella, et al. and applied to the uptake of a single protein[13] and to the competitive adsorption of proteins.[14] The theory of Yigit et al. considers only weakly charged networks.[13] Hence, the electrostatic interaction was mainly determined by the monopole term deriving from the Donnan-potential of the network and the net charge of the protein.[13] The Born energy that describes the solvation of a charged protein in solutions of different ionic strength was added as a further term to the free energy of binding. The comparison with experimental results demonstrated

U. Freudenberg, P. Atallah, J.-U. Sommer, C. Werner
Institut Biofunktionelle Polymermaterialien
Leibniz-Institut für Polymerforschung Dresden e.V.
Hohe Strasse 6, D-01069 Dresden, Germany
J.-U. Sommer
Institut für Theoretische Physik
TU Dresden
Zellescher Weg 17, D-01069 Dresden, Germany
J.-U. Sommer, C. Werner
Cluster of Excellence Physics of Life
TU Dresden, D-01307 Dresden, Germany
M. Ballauff
Institute of Chemistry and Biochemistry
Freie Universität Berlin
Takustr. 3, D-14195 Berlin, Germany
E-mail: matthias.ballauff@fu-berlin.de

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mabi.202200561

DOI: 10.1002/mabi.202200561

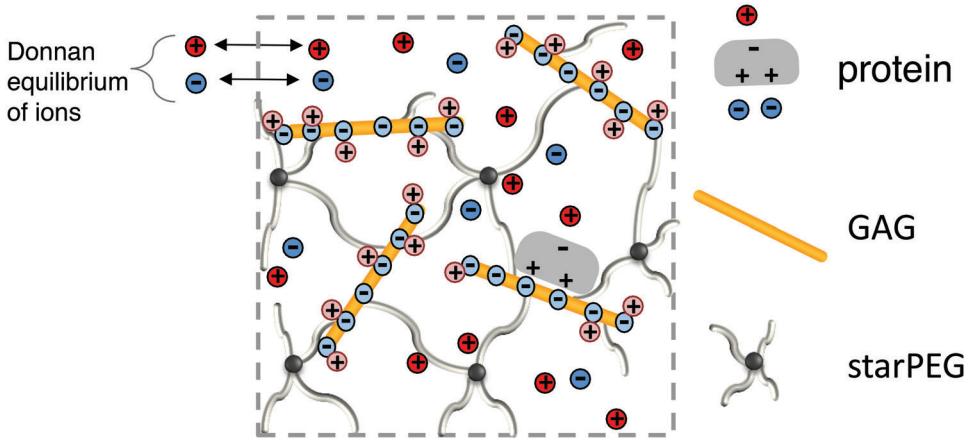

Figure 1. Scheme of a highly charged and hydrated polymer network consisting of two building blocks, namely, uncharged, star-shaped poly(ethylene glycol) (starPEG) and negatively charged glycosaminoglycans (GAG) (see the details in ref[38]). The GAG-chains are crosslinked by the tetrafunctional polyethylene glycol stars (starPEG) as it has been described in detail in ref[38] where all experimental work has been performed. Three equilibria are considered: i) The concentration of the anions and cations inside the network are adjusted by a Donnan-equilibrium with the concentration in the reservoir; ii) Cations which are condensed to the charged segments ([IMAGE]; marked in light red) within the network are in equilibrium with the free cations ([IMAGE]; marked in dark red) in the network; and iii) The proteins in the reservoir are in equilibrium with the proteins bound to the network. The positive surface patches of the proteins interact closely with the highly charged GAG-chains thus releasing a concomitant number of counterions. The entropic gain by this process is an important driving force for binding of proteins to a such highly charged networks.

that this theory needs just one adjustable parameter to describe the binding constant.[13,14] With this parameter which was explained by hydrophobic interaction, theory could predict the competition of two proteins on such a weakly charged network with remarkable success.[14] If, however, the distance between charges along the polymer chains b is less than the Bjerrum-length l_B ($l_b = e^2/4\pi\epsilon_0\epsilon kT$; e: elementary charge; ϵ_0: permittivity of vacuum; ϵ: dielectric constant; k: Boltzmann constant; T: temperature; l_B = 0.71 nm for water at 25 °C)[16] counterion condensation sets in.[16] In this case, the charge parameter $\xi = l_B/b$ of the polymer chains of the network is larger than unity, and a fraction of the counterions given by 1-1/ξ will be condensed to these chains.[16,17] Hence, these condensed counterions will no longer contribute to the osmotic pressure in the system which must be taken into account for the calculation of the Donnan-potential as well. The swelling equilibria in such networks must hence use the effective charge density of the chains given by the fraction of free charges without condensed counterions.[18,19] To the authors' best knowledge, there is no treatment yet for the protein adsorption to such highly charged networks.

Complex formation of proteins with highly charged free polyelectrolytes, on the other hand, is a long-standing and rather well-understood problem of research.[5,20–24] Proteins can be bound to such polyelectrolytes and this interaction can lead to phase separation at higher concentrations ("complex coacervate"; cf. ref.[20, 22, 25]). Recent work has shown that the binding of proteins to highly charged polyelectrolytes characterized by a charge parameter $\xi > 1$ is mainly determined by counterion release: Patches of positive charge on the surface of the protein will become a multivalent counterion of the polyelectrolyte thus releasing a concomitant number Δn_{ci} of counterions.[26–30] A review of this work and possible medical applications has been presented recently.[31] Hydrophobic interaction, namely, the release or uptake of water molecules during complex formation can be included in this model as well.[32,33] It rests to extend these considerations to a model that would allow us to understand the interaction of highly charged networks with proteins.

Here we develop a theoretical model that is capable of describing the interaction of proteins with highly charged networks. The study is motivated by the fact that biomolecular signals in living matter are modulated by the interplay of globular proteins with highly charged polymer structures, designated as sulfated glycosaminoglycans (GAG), as they appear within the glycocalyx of the cell or the extracellular matrix (ECM) as, e.g., in the glycocalyx of the cell.[34–37] Moreover, charged hydrogels can be used for medical applications to sequester or release signaling molecules.[38] Thus, sulfated glycosaminoglycan-based hydrogels with varied GAG content and GAG sulfation pattern were prepared and applied to sequester cytokines for resolving inflammation and thereby supporting wound healing.[39–44] GAGs are highly charged polymers that constitute important components of the ECM.[34–37]

The aim of the present work is a fully quantitative understanding of the experimental data given in ref [38] for protein interactions with different GAG-based hydrogels. **Figure 1** displays the overall structure of the hydrogels studied in ref.,[38] GAGs such as heparin or selectively desulfated heparin are crosslinked with 4-arm star-shaped poly(ethylene glycol) (starPEG) to form covalent networks.[38] For simplification details of the crosslinking chemistry and the particular properties of the respective building blocks have been omitted in Figure 1 but are given in ref.[38] The architecture of these networks allows us to vary the decisive parameters of such systems within wide limits: First of all, the number of GAG-units within the network can be adjusted precisely. The overall concentration of charged units c_p is thereby fixed and the concentration of GAG-chains and with this the concentration ***P1*** of charges in swollen networks is known accurately. Parameter **P1** hence could be varied over a wide range from 0.001 to 0.12 mmol ml^{-1}. The GAG-units used to form the network can be selectively desulfated so that the second parameter ***P2***, the

Table 1. Molecular weight and isoelectric points of the cytokines used in the experimental study of protein adsorption to GAG-networks.[38] The concentration $c_{prot,0}$ is the overall concentration of a given protein used in the adsorption experiments. Uniprot ID: cf. www.uniprot.org.

Protein class	Name	*M*wt [kDa]	IEP	$c_{prot,0}$ /*pM*	Uniprot ID
A	VEGF-A	38.2	9.2	0.097	P15692
	bNGF	13.5	9	0.302	P01138
	IL-8	8.9	9.2	0.233	P10145
	MCP-1	8.7	9.4	0.391	P13500
B	MIP-1 alpha	7.7	4.8	0.244	P10147
	MIP-1 beta	7.8	4.8	0.634	P13236
C	IL-6	20.8	6.2	0.230	P05231
	TNF-alpha	17.4	7	0.246	P01375
D	EGF	6.2	4.8	0.275	P01133
	GM-CSF	14.5	5.2	0.478	P04141

number of sulfate groups per GAG-unit in the network divided by the molecular weight of the repeating unit can be adjusted independently from parameter ***P1***. Parameter **P2** directly defines the charge parameter ξ by adjusting the number of charges per repeating unit. Evidently, both parameters fully characterize these networks: Parameter ***P1*** gives the overall charge density in the swollen state. If parameter ***P2*** is small, the adsorption of proteins to such weakly charged networks can be treated in terms of the theory developed by Yigit and Dzubiella.[13,14] If, however, parameter ***P2*** is increased, the charge parameter ξ will exceed unity and the chains of this highly charged network will interact with proteins mainly via the counterion release mechanism.[17,31,32] It should be noted that details of the crosslinking procedure nor the polymer network architecture does not matter for the outcome of the subsequently discussed theoretical modeling of protein interactions with the hydrogels as long as two design criteria are fulfilled: (I) Sterically restrictions to protein transport should be neglectable and (II) the charge related parameters P1 and P2 should be precisely controllable but satisfying in parallel before mentioned sterically restrictions. Given the fact that the networks used in ref.[38] have a mesh size of 10 to 13 nm in the swollen state which is much larger than the typical dimensions of the proteins that have been studied the (I) criteria is fulfilled. We thus deal with highly hydrated polymer networks (i.e., hydrogels) in which possible steric interactions or diffusional barriers do not play any role for the adsorption of proteins. Furthermore, the synthesis strategy that have been used in ref.[38] for the experimental work did allow to precisely and independently control of the charge parameters **P1** and **P2** over a broad range.

Using these networks characterized by different parameters **P1** and **P2**, adsorption studies with mixtures of biomedically important cytokines with different surface charge characteristics were conducted.[38] **Table 1** gathers the molecular weight and the isoelectric points of the cytokines used in this study. Cytokines grouped into (A) are strongly basic proteins with an isoelectric point (IEP) $\geq$ 9.0. Cytokines in group (B) are overall acidic proteins (IEP 4.8) that possess a positively charged heparin-binding domain. Group (C) contains weakly charged or neutral proteins of an IEP between 6 and 7. Finally, group (D) gathers acidic proteins with an IEP smaller than 5.5 that display no heparin-binding domain. Adsorption experiments were done as follows[38]: Micron-sized slices of defined GAG-hydrogels were equilibrated with highly diluted mixtures of these cytokines in a buffer.[38] The salt concentration was set in all cases to 150 mM to match physiological conditions. Table 1 gathers the concentrations of the respective proteins within the mixture. The degree of adsorption was determined by analyzing the composition of the supernatant after 24 h of contact using a multiplex assay. In this way, the sequestration of medically important cytokines by GAG-hydrogels could be determined quantitatively. Further experimental details are given in ref.[38] and further papers cited therein.

We compare these experimental data[38] to a simple semi-quantitative theory of protein adsorption on highly charged networks developed here. For this purpose, we combine the theory of Yigit and Dzubiella[13] with recent work on the interaction of free polyelectrolyte chains with proteins.[30,32,45] The main goal of the present work is an understanding and modeling of the forces that lead to the adsorption of different proteins to the polymer network.

1.1. Theory

We start with the definition of the system as given in Figure 1, We consider a gel composed of negatively charged chains immersed in an aqueous solution of monovalent ions with concentration c_s^b. For the sake of simplicity, we assume that the charged moieties of the network are fully dissociated. Proteins are modeled as spheres with radius R_p and net charge number z_p that results from the balance of number N_- of negative and N_+ of positive charges on its surface. The number of charged units N_g per gel volume V_{gel} defined the charge concentration **P1** within the gel. For a direct comparison with the systems depicted in Figure 1 the charge within the network comes solely from the known number N_{GAG} of GAG chains each of which carries n_{GAG} charges. Therefore, we have for the concentration of charges within the network $\mathbf{P1} = c_p = n_{GAG}N_{GAG}/V_{gel}$. These chains are highly charged, that is, the average distance between the charges along the contour of the chain is smaller than the Bjerrum length $l_B \cong 0.7$ nm. For the fully sulfated repeating unit of heparin a charge parameter $\xi = 2.84$ was found.[46] The analysis given there furthermore showed that the carboxyl-group of heparin is fully charged at neutral pH.[46] The network is immersed in a reservoir with the concentration $c_{prot,i}$ of a given protein in a buffer at physiological salt concentration.

The present model disregards any steric interaction of the proteins with the network. In principle, the uptake of a protein requires work against the osmotic pressure of the network that can be treated in terms of a semi-dilute polymer solution (see the discussion of this point in ref.[47]). Hence, the free energy of the uptake of a single protein with volume V_{prot} will scale as $V_{\mathrm{prot}}/\xi_{\mathrm{corr}}{}^3$ where ξ_{corr} is the correlation length of the polymers in the network. The length ξ_{corr} can be estimated to be between 4 and 9 nm whereas the proteins under consideration here are small and have dimensions of $\approx$2 nm only. Therefore, the contribution due to steric interaction will be of the order of kT at the most and can be safely dismissed. In addition to this, the excluded

volume interaction among the proteins is disregarded since it comes into play only at high protein concentrations within the network.[13,14,48] Also, the network is assumed to be entirely homogeneous so that boundary effects due to a surface layer can be disregarded. The electric field would be finite in such a boundary layer and its interaction with the dipole moment of the protein[49] would come into play as discussed recently by Androher-Benitez et al.[48]

As shown in Figure 1 we have to consider the following equilibria:

i. The Donnan-equilibrium between the ions inside and outside the network. For a given salt concentration c_s^b outside the network we require the concentration c_s^g of the ions that penetrate the network from outside.
ii. Counterion condensation will take place on the highly charged GAG-segments within the network until the effective charge parameter is unity, exactly in the sense of Manning's theory.[13] Hence, a part of cations $1 - 1/\xi$ within the network will be bound firmly to the charged segments and will not contribute to the osmotic pressure.[18]
iii. The interaction of proteins with the polymer network can now be described with two terms: First, the protein is treated as an entity having a given net charge z_p that interacts with the network exactly as lined out in ref.[13, 14] (see the details below). Second, there will be an interaction of the proteins with the highly charged GAG-sequences by counterion release.[5,27] The driving force for this interaction is defined by the ratio of the concentration of the cations condensed to the GAG-sequences to the concentration of cations resulting from the Donnan-equilibrium.

i) Donnan-equilibrium: The Donnan-equilibrium of networks has been considered in terms of ideal ions inside and outside of the network (cf. the review of Quesada-Perez[19]). However, the ions inside of a network move in the electric field of the charged polymer chains which will lower their activity. This problem has first been considered by Katchalsky and Michaeli in 1955 who developed analytical corrections for the classical Donnan-term.[2] Recently, the activity of ions within charged networks has been re-considered in great detail by Kosovan, Holm, and their coworkers.[4,50–53] The main point of this model is the fact that the salt concentration c_s^g within the gel is much higher than anticipated by the classical Donnan-model. This is due to the marked decrease in the activity of the counterions because of their strong interaction with the chains of the network. For the present purpose of a first semi-quantitative discussion of protein adsorption to GAG-networks obtained at high ionic strength,[38] the classical Donnan-equilibrium suffices and corrections for the higher ion concentration within the network may be added in a later stage.

Within the classical Donnan-model, the ratio r of the concentration of salt ions that enter the gel c_s^g to the respective bulk concentration c_s^b follows as[2,50]

$$r = \frac{c_s^g}{c_s^b} = \left[\left(\frac{c_p}{2c_s^b}\right)^2 + 1\right]^{1/2} - \frac{c_p}{2c_s^b} \tag{1}$$

1.2. The Donnan-Potential $\Delta\phi$ Scaled by e/kT is Given by

$$\Delta\widetilde{\Phi} \equiv \frac{e}{kT}\Delta\Phi = \ln r \tag{2}$$

Since we consider the uptake of small amounts of protein compared to the total charge of the polymer network **P1** only, shifts of the ion concentration within the network by the adsorbed proteins as discussed in ref.[13] can be safely neglected. With the experimental degree of swelling and the parameter **P1** referring to the swollen network, c_p = **P1** and Equation 1 can be used to calculate the ratio r.

ii) Counterion condensation: For the highly charged GAG-segments in the network, the charge parameter ξ exceeds unity. Hence, a part of the ions within the network will condense onto these segments until the charge parameter is unity again. Using Manning's theory[13] the charge density c_p provided by the GAG-sequences will therefore be reduced to c_p/ξ. To account for this effect, Equation (1) must be changed to[18,19]

$$r = \left[\left(\frac{c_p/\xi}{2c_s^b}\right)^2 + 1\right]^{1/2} - \frac{c_p/\xi}{2c_s^b} \tag{3}$$

iii) Uptake of protein; weakly charged networks: For the free energy of the uptake of a protein into this network, we first consider the contribution already operative in a weakly charged network. Here Yigit et al. could show that the difference of free energy between a protein outside and inside a weakly charged gel can be well approximated as follows[13]

$$\frac{\Delta G_{el}}{kT} = z_p\Delta\widetilde{\phi}\left(c_s^b\right) - \frac{z_p^2 l_B}{2R_p}\left(\frac{\kappa_g R_p}{1+\kappa_g R_p} - \frac{\kappa_b R_p}{1+\kappa_b R_p}\right) \tag{4}$$

where κ_g and κ_b are the inverse screening lengths in the gel and the bulk, respectively. The first term is the monopole term that depends on the sign of the z_p. For cationic proteins, it is negative and presents an unspecific driving force for the uptake into the negatively charged gel. The second term is the Born term related to the screening of the surface charge of the proteins. This term denotes the gain of free energy when transferring a charged sphere of radius R_p from a medium of salt concentration c_b^b to a medium with salt concentration $c_p/\xi + c_s^g$. The inverse screening length κ_g in the gel then follows as[13]

$$\kappa_g = \left(8\pi l_B(c_p/\xi + c_s^g)\right)^{1/2} \tag{5}$$

Hence, the two terms in Equation 4 describe the transfer free energy when a protein enters into a weakly charged network.

Uptake of protein; strongly charged networks: As illustrated in Figure 1, a second specific interaction takes place in case of highly charged networks: The positive patches on the surface of the protein interact with the charges on the GAG-chains of the polymer network thereby releasing a number Δn_{ci} of counterions condensed to these highly charged sections of the chains. In principle, this is the interaction of a protein with a free linear polyelectrolyte chain in solution studied in detail in a number of previous investigations.[5,26,27,29,30,32,33,46,54] The resulting free energy of binding ΔG_b^{free} can hence be determined precisely for various salt

concentrations c_s^b . In general, the dependence of ΔG_b^{free} on the concentration c_s of a monovalent salt can be rendered as[45,55]

$$\Delta G_b^{\text{free}} = RT\Delta n_{ci} \ln c_s^b - RT0.036\Delta w c_s + \Delta G_{\text{res}} \quad (6)$$

where Δn_{ci} is the net number of released counterions whereas Δw denotes the net effect of water release/uptake during binding as discussed recently.[45] This first term will only come into play when the charge parameter ξ is larger than unity, that is, only if counterion condensation takes place on the chains of the network. Since the number of condensed counterion is given by 1-1/ξ, the effect of counterion release will scale as 1-1/ξ for a given protein. The second term in Equation 6 containing Δw is usually quite small[32,33] and defines hydrophobic interaction. ΔG_{res} denotes the residual free energy of binding resulting for a salt concentration of 1 M.[30,32] According to previous simulations this term refers to the interaction of the polyelectrolyte and the protein at direct contact through salt bridges and/or hydrogen bonding,[30] i.e., together with Δw it can be attributed to rather short-range specific interactions between the partners. In the following, we assume that both free energies as given by Equation 4 and Equation 6 add up

$$\Delta G_b = \Delta G_{el} + \Delta G_b^{free} = -RT \ln K_b \quad (7)$$

where K_b denotes the thermodynamic binding constant. Hence, the total free energy consists of two terms that react differently to the environment which is mainly defined by c_s^b.

1.3. Evaluation of the Experimental Binding Constant

The experiment conducted by Freudenberg et al.[38] measured the depletion of a given cytokine out of a mixture of in total 16 proteins. Table 1 gathers the total concentrations $c_{\text{prot},0}$ of the cytokines in the mixtures used in the adsorption experiments. The data thus obtained can be evaluated in terms of the competitive Langmuir adsorption isotherm where each chain in the network has N binding places from which $N_{b,i}$ are covered at a certain stage by protein i. Therefore the coverage θ_i for a protein of sort i is defined by

$$\theta_i = \frac{N_{b,i}}{N} \quad (8)$$

For the Initial Protein Concentration $c^i_{\text{prot},0}$ of Protein I, we have

$$c^i_{\text{prot},0} = c^i_{\text{prot},free} + N\theta_i c_{GAG} \quad (9)$$

The Langmuir Adsorption Isotherm Reads

$$\theta_i = \frac{K_{b,i} c^i_{\text{prot},free}}{1 + \sum K_{b,i} c^i_{\text{prot},free}} \cong K_{b,i} c^i_{\text{prot},free} \quad (10)$$

since $\sum K_{b,i} c^i_{\text{prot},free} \ll 1$ for the experimental conditions used in ref.[38] The degree of coverage θ is given by

$$\theta_i = \frac{c^i_{\text{prot},0} A}{N\, c_{GAG}} \quad (11)$$

with c_{GAG} being the total concentration of the GAG-chains in the network and A is the amount of bound protein of a given protein in percent. The binding constant K_b follows as

$$K_{b,i} = \frac{\theta_i}{c^i_{\text{prot},free}} = \frac{c^i_{\text{prot},0} A}{N\, c_{GAG}\,(100 - A)\, c^i_{\text{prot},0}} = \frac{A}{N\, c_{GAG}\,(100 - A)} \quad (12)$$

Here it is assumed that the number of free GAG-chains can be safely equated to the total number of GAG-chains because of the very low coverage θ.

2. Results and Discussion

The main point of the present analysis is embodied in Equation 7. Accordingly, the free energy of binding ΔG_b consists of the free energy ΔG_b^{free} of binding of the protein to a single free polyelectrolyte chain and is related to counterion release effects and specific interactions. The term ΔG_{el} (Equation 4) is due to the embedding of these polyelectrolyte chains and the protein in the polymer network. The latter expression is dominated by the monopole term $z_p \Delta\widetilde{\phi}\,(c_s^b)$ which depends on the charge of the chains within the network which thus can be positive or negative. The second term in Equation 4 is always negative since the salt concentration within the network will always be equal or larger than the salt concentration outside, i.e., it will always result in an attractive contribution for protein binding. The free energy ΔG_b^{free} of protein binding to free polyelectrolyte chains, on the other hand, will be negative in practically all relevant cases if the salt concentration within the network is small. In this case, it may dominate and overcome the monopole term in Equation 4, so that adsorption of negatively charged proteins to anionic networks becomes possible. A similar situation arises for negatively charged polyelectrolyte brushes which may adsorb proteins above their isoelectric point.[5,56] For larger salt concentrations inside the network, on the other hand, ΔG_b^{free} may become too small and as a result no adsorption will take place.

For cationic proteins of type A (Table 1), two cases can be distinguished:

i. The outside salt concentration c_s^b is small. Then the concentration c_s^gwill be small and the salt concentration inside the gels is more or less identical to the concentration c_p defined by the number of charged units. Therefore both ΔG_{el} and the term ΔG_b^{free} will become dominant. Strong adsorption of proteins will take place regardless of the charge distribution on the protein surface.
ii. The gel is immersed into a solution with high salt concentration ($c_s^b \approx 150$ mM). Then ΔG_{el} will be much smaller since c_s^g is of the order of c_s^b. A Donnan-exclusion, that is, $c_{s,-}^g$ is considerably smaller than c_s^b, may occur, however, if the concentration c_p of charged units with the network is of the order of c_s^b. It must be noted that only the effective charge density c_p/ξ comes into play (see Equation 3). Thus, if a high charge density is induced by chains with $\xi > 1$, the resulting Donnan-exclusion as expressed through the lowering of c_s^g and with this the magnitude of ΔG_{el} (Equation 4) will be greatly diminished. Hence, ΔG_b will be dominated by ΔG_{free} which will be

Table 2. Survey of the experimental results on the adsorption of proteins to networks.

	Gel type	1: PEG-HEP-1		2: PEG-NDHEP-2		3: PEG-HEP-3		4: PEG-HEP-4		5: PEG-6ONDHEP-5		6: PEG-HEP-6	
	P1/P2	114.5/0.005		112.7/0.0035		112.7/0.005		75.1/0.005		61.58/0.002		35.58/0.005	
	C_{GAG}/ξ	1.69/2.84		2.41/1.93		1.67/2.84		1.11/2.84		2.63/0.97		0.526/2.84	
		A	K_b 10^{-3}	A	K_b 10^{-3}	A	K_b 10^{-3}	A	K_b 10^{-3}	A	K_b 10^{-3}	A	K_b 10^{-3}
A	VEGF-A	90 ± 1	5.31	86 ± 1	2.55	71 ± 4	1.47	55 ± 5	1.11	69 ±4	0.925	34 ± 8	0.98
	bNGF	84 ± 7	3.10	79 ± 4	1.56	93 ± 1	7.97	71 ± 8	2.21	71±4	1.02	54 ± 9	2.23
	IL-8	94 ± 1	9.24	70 ± 2	0.969	79 ± 1	2.26	71 ± 7	2.21	45 ± 3	0.340	55 ± 6	2.32
	MCP-1	95	11.21	88 ± 1	3.05	81 ± 1	2.56	70 ± 9	2.10	45 ± 4	0.339	51 ± 8	1.98
B	MIP-1alpha	58 ± 3	0.815	41 ± 1	0.288	67 ± 2	1.22	53 ± 5	1.02	21 ± 4	0.11	35 ± 3	1.02
	MIP-1beta	56 ± 2	0.751	38 ± 3	0.255	66 ± 2	1.16	49 ± 7	0.87	23 ± 3	0.124	32 ± 3	0.89
C	IL-6	24 ± 5	0.186	8 ± 5	0.036	61 ± 1	0.938	42 ± 4	0.65	17 ± 8	0.085	22 ± 5	0.536
	TNF-alpha	39 ± 3	0.377	25 ± 3	0.138	56 ± 4	0.763	46 ± 6	0.767	25 ± 4	0.138	26 ± 4	0.668
D	EGF	7 ± 5	0.044	8 ± 3	0.036	24 ± 3	0.190	12 ± 2	0.123	11 ± 1	0.051	7 ± 3	0.143
	GM-CSF	6 ± 2	0.37	4 ± 1	0.017	27 ± 1	0.22	16 ± 2	0.172	11 ± 3	0.051	6 ± 3	0.121

P1: charge density in swollen gel in mM (mmol L^{-1}), C_{GAG} in mmol L^{-1}

smaller but still of appreciable magnitude when compared to case i). In this way, the polyelectrolyte chains of the network interact with the proteins much in the way of free chains.

For proteins of type B, the Donnan-term ΔG_{el} will be positive since the overall charge of the proteins is negative as well. However, the heparin-binding domain will strongly interact with the chains within the network which is followed by a negative contribution ΔG_{free} which ultimately can lead to binding of the protein. In addition to these terms due to electrostatic interaction, hydrophobic interaction as embodied in the second term of Equation 6 may lead to protein adsorption even in cases where the electrostatic attraction is not operative anymore. This effect will be operative for the proteins of class C and D and may lead to binding in absence of electrostatic effects.

Table 2 summarizes the main results on the interaction of the well-characterized GAG-networks[57] with different cytokines.[38,42,58–60] Here we evaluate the data of six networks differing in charge density **P1** and parameter **P2** defining the charge parameter ξ of the chains. System PEG-HEP-1 possesses the repeating unit of heparin with the highest charge density leading to a charge parameter $\xi = 2.84$ (see the second row of Table 2). The calculation of ξ has been done as lined out recently[46]: The repeating unit of heparin has a length of 1 nm and bears 4 charges. The length b per charge is therefore 0.25 and $\xi = l_B/b = 2.84$. The other systems bear less charges per repeating unit which leads to a concomitantly smaller **P2** and ξ. The other decisive parameter is c_{GAG}, the molar concentration of heparin or modified heparin chains per volume. Hence, these systems allow us to change the overall charge density **P1** by considering systems 1, 3, 4, and 6 while keeping parameter **P2** and thus ξ constant. On the other hand, comparing results from systems 1, 2, and 5 will show mainly the influence of the local charge density as expressed through ξ. The concentration c_{GAG} and parameter **P1** refer to the swollen networks and can directly be used to calculate the Donnan-equilibrium according to Equation 4.

To these networks mixtures of cytokines of type A to D (cf. Table 1) have been added.[38] The concentrations of the cytokines in these solutions are extremely small (cf. Table 1) which justifies the approximation made in Equation 10. The depletion of a given cytokine in solution after equilibration with the network was measured which leads to the amount of protein adsorbed to the network. Table 2 gathers the percentage A of adsorbed protein for a given network. Equation 12 then leads to the binding constant K_b tabulated for each combination of a network and a given cytokine (cf. Table 2). In this calculation, the number of adsorption sites N per chain in the network is not known. However, we can compare the present system to our recent experiments in which the binding of lysozyme to heparin has been analyzed.[46] Previous work has demonstrated that lysozyme is a good model for cytokines.[27] For free heparin chains, we found that ≈6 lysozyme molecules are bound at a heparin chain of molecular weight 16 000.[46] Since N cannot be evaluated from the present experiments for a given network and protein, we set this parameter to unity in all calculations presented here. Evidently, Equation 10 works best for A-values between 10% and 90%. For stronger or weaker degrees of adsorption, the experimental error of K_b will become too high, and data deriving from these data will not be considered in the subsequent discussion. Hence, only a part of the data given in ref.[38] could be evaluated here. The binding constants K_b summarized in Table 2 may then be converted to the binding free energy ΔG_b via Equation 7.

The values for cytokines of type A and the respective free energies of binding gathered in Table 2 are much smaller than anticipated from data obtained for heparin/lysozyme extrapolated to a salt concentration of 0.15 M.[46] A direct comparison can be done for VEGF-A where the binding constant with heparin and heparin oligomers had been measured by Zhao et al.[61] The binding constant K_b for VEGF/heparin was found to be of the order of 10^7 M. Smaller values down to 10^5, however, are found for heparin-oligomers. This finding points to the fact that a minimum length of heparin oligomers is necessary for the electrostatic binding of proteins.[62]

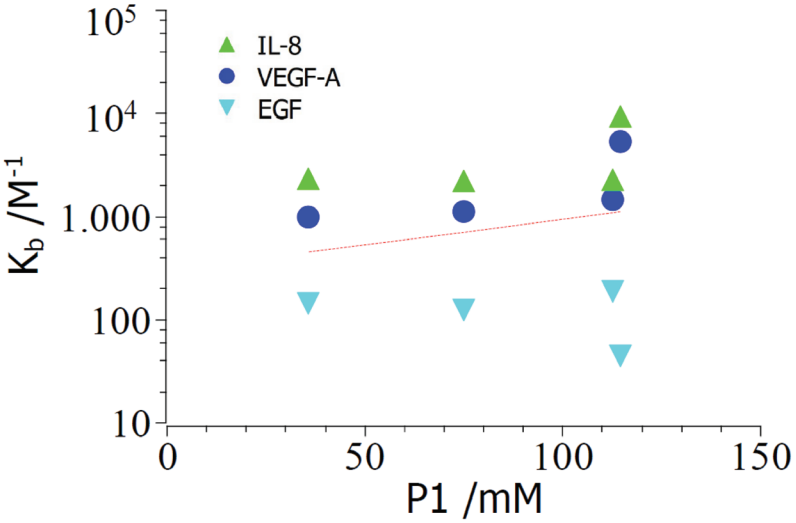

Figure 2. The binding constant K_b obtained for IL-8, VEGF-A, and for EGF is plotted against the charge density P1 in the network while keeping the charge parameter ξ constant. The data have been derived (from left to right) for systems 1, 3, 4, and 6 (cf. Table 2). The line shows the model calculation for the binding constant based on Equation 7. See text for further explanation.

The different data may first be discussed by comparing cytokine adsorption to polymer networks 1, 3, 4, and 6 where the overall charge density **P1** is being changed systematically while keeping parameter **P2** and thus $\xi = 2.84$ constant. In this way mainly the influence of the term ΔG_{el} in Equation 7 is probed. The parameter Δn_{ci} in Equation 6 remains constant because it is strongly dependent on the charge parameter ξ.

Figure 2 explore the consequences of the charge density of the network by showing the binding constant K_b as the function of **P1**. The data have been derived (from left to right) for systems 6, 4, 3, and 1 (cf. Table 2). Here the results for just three proteins are shown, namely for IL-8 and VEGF-A which are proteins of class A (see Table 1) and EGF which belongs to class D. The binding constant for proteins of type A are highest and decreases subsequently for classes B to D. A similar trend is observed for the other proteins from class A, namely, K_b is decreasing with decreasing **P1** while the charge parameter is kept constant. The effect is small, though outside of experimental error.

In order to understand the effect of ΔG_{el} in more detail, a simple model calculation is done: For a temperature $T = 298$K we can estimate ΔG_b^{free} to be of the order of 5 – 6 kT. This magnitude can be derived from the first term of Equation 6 assuming that 3 counterions are released during binding into a solution with a salt concentration of 0.15 M. Hence, $\Delta G_b^{\text{free}}/kT = -5.69$. $\Delta G_{el}/kT$ can be estimated from Equation 4 with neglect of the Born-term to be – 1.33 for system 1 (see Table 2). A concomitantly smaller value follows for systems 3, 4, and 6. The red line in Figure 2 indicates the resulting binding constant K_b. The changes of K_b seen in the experimental data for VEGF-A are comparable to the changes effected by the Donnan-potential. However, the present limits of error do not allow us to do a fully quantitative comparison.

Similar observations can be made for all proteins of class A indicating a small but finite contribution from ΔG_{el}. For the proteins of class B this effect can no longer be observed but the overall negative charge will lead to a repulsive monopole term (first term in Equation 4). The same holds true for proteins of classes C and D.

A comparison of the data obtained with systems 1, 2, and 5 allows us to check the influence of the charge parameter ξ. System 5 has a slightly lower charge density **P1** as systems 1 and 2. However, the foregoing discussion has revealed that the influence of charge density is rather small, except for system **1**. To a first approximation, we can rationalize the binding constant K_b for a varying charge parameter ξ in the following way: As already argued above, a fraction 1-1/ξ of the counterions will be condensed to the chains.[16] Hence, this fraction will increase the concentration near to the macroion considerably. Manning has argued that this increase of concentration can be captured in terms of a surface concentration c_{ci} which is linked to the number of condensed counterions.[17] The concept of a surface concentration due to counterion condensation has been corroborated in recent MD-simulations[63] and found to be very useful when considering the binding of proteins to highly charged polyelectrolytes.[5,27] Thus, protein binding to polyelectrolyte chains should correlate with the fraction of condensed counterions.

Figure 3 shows the binding constant K_b against 1-1/ξ. The strong dependence of the free energy of binding on the charge parameter for proteins of group A is directly obvious. MIP-1alpha belonging to protein group B exhibits a much weaker dependence since opposite effects with charge-driven repulsion and counterion release are present (see discussion above for Table 2). K_b obtained for proteins in groups C and D is independent of ξ within the limits of error. As an example of the proteins of these groups, we plot here the data for EGF. The data shown in Figure 3 underscore directly the importance of the counterion release mechanism: For $\xi < 1$, no counterion condensation takes place, and the first term in Equation 6 vanishes. Only if ξ is significantly larger than unity, counterion condensation sets in, and a fraction 1-1/ξ of the counterions is bound firmly to the macroion. Figure 3 demonstrates the consequences of the increasing charge parameter directly in a semi-logarithmic plot of K_b against 1-1/ξ:

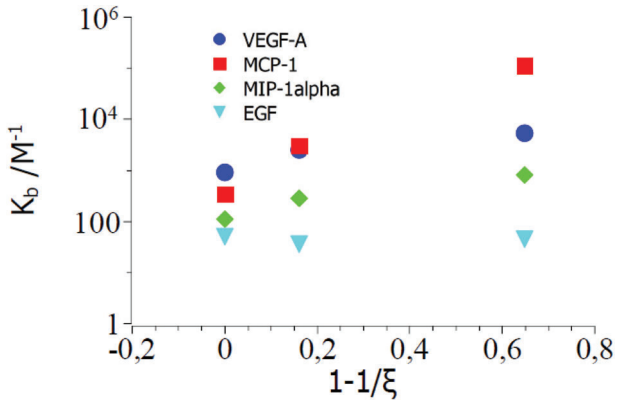

Figure 3. Analysis of the dependence of the binding constant on the charge parameter ξ as measured for systems 5, 4, and 1 from left to right. The binding constant K_b is plotted against 1-1/ξ of the respective systems (cf. Table 2) that is, against the fraction of condensed counterions according to Manning's theory.[16] See text for further explanation.

For proteins of type A, there is a clear correlation of the free energy of binding to the fraction of condensed counterions. A slightly weaker increase of K_b with 1-1/ξ is found for cytokines of type B, here exemplified for MIP-1alpha. No correlation is found for proteins of type D which was already apparent from the discussion in Figure 2.

3. Conclusions

A simple model of protein binding to strongly charged networks has been developed. Two main factors lead to adsorption: i) the Donnan-potential of the network leading to Equation 4, and ii) the counterion release effect (see Equation 6) that is already operative for free chains. Both contributions add up and the entire free energy Equation 7 can be determined quantitatively for a given network. This model is used to discuss the data obtained previously for the adsorption of cytokines to highly charged GAG-networks.[38] The model can explain all trends seen in these experiments and in particular justify the classification of these proteins into classes A – D (Table 1): Markedly positive cytokines of class A will adsorb much stronger than the ones of class B and C. For class D, virtually no influence of charge-charge interaction is seen and no binding to the polymer networks occurred. Viewed together, the comparison of the model with experiments demonstrates that charge-charge interaction can raise the binding constant by 2–3 orders of magnitude.

The present comparison demonstrates that a semi-quantitative understanding and prediction of the binding of proteins to highly charged networks can be achieved based on the classification of proteins as is done in Table 1. Since the mechanism of protein/polyelectrolyte interaction in a polyelectrolyte brush layer is very similar to the one discussed here,[48,49] this classification will be highly useful when considering the interaction of proteins with brush-like systems as, e.g., the glycocalyx or the ECM. Accordingly, the results could pave the way for a deeper understanding of how these important signaling molecules are managed in tissues and thus help design artificial matrices to control cell fate decisions.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 434130070, GRK 2662, was gratefully acknowledged. MB was indebted to Joachim Dzubiella for helpful discussions.

Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

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

Keywords

counterion release, Donnan-equilibrium, networks, polyelectrolyte, proteins, protein adsorption

Received: December 22, 2022
Revised: March 17, 2023
Published online: April 26, 2023

[1] P. J. Flory, *Principles of Polymer*, Chemistry, Cornell University Press, Ithaca **1953**.
[2] A. Katchalsky, I. Michaeli, *J. Polym. Sci.* **1955**, *15*, 69.
[3] P. Kosovan, T. Richter, C. Holm, *Macromolecules* **2016**, *49*, 2409.
[4] O. Rud, T. Richter, O. Borisov, C. Holm, P. Kosovan, *Soft Matter.* **2017**, *13*, 3264.
[5] X. Xu, S. Angioletti-Uberti, Y. Lu, J. Dzubiella, M. Ballauff, *Langmuir* **2019**, *35*, 5373.
[6] Y. Li, R. de Vries, T. Slaghek, J. Timmermans, M. A. C. Stuart, W. Norde, *Biomacromolecules* **2009**, *10*, 1931.
[7] Y. Li, R. D.e Vries, M. Kleijn, T. Slaghek, J. Timmermans, M. C. Stuart, W. Norde, *Biomacromolecules* **2010**, *11*, 1754.
[8] Y. A. Li, J. M. Kleijn, M. A. C. Stuart, T. Slaghek, J. Timmermans, W. Norde, *Soft Matter.* **2011**, *7*, 1926.
[9] Y. Li, Z. Zhang, H. P. Van Leeuwen, M. A. Cohen Stuart, W. Norde, J. M. Kleijn, *Soft Matter.* **2011**, *7*, 10377.
[10] N. Welsch, J. Dzubiella, A. Graebert, M. Ballauff, *Soft Matter.* **2012**, *8*, 12043.
[11] S. Angioletti-Uberti, M. Ballauff, J. Dzubiella, *Soft Matter.* **2014**, *10*, 7932.
[12] S. Matsui, K. Hosho, H. Minato, T. Uchihashi, D. Suzuki, *Chem. Commun.* **2019**, *55*, 10064.
[13] C. Yigit, N. Welsch, M. Ballauff, J. Dzubiella, *Langmuir* **2012**, *28*, 14373.
[14] M. Oberle, C. Yigit, S. Angioletti-Uberti, J. Dzubiella, M. Ballauff, *J. Phys. Chem. B* **2015**, *119*, 3250.
[15] S. Angioletti-Uberti, M. Ballauff, J. Dzubiella, *Mol. Phys.* **2018**, *116*, 3154.
[16] G. S. Manning, *J. Chem. Phys.* **1969**, *51*, 924.
[17] G. S. Manning, *Q. Rev. Biophys.* **1978**, *11*, 179.
[18] B. A. Mann, C. Holm, K. Kremer, *J. Chem. Phys.* **2005**, 122.
[19] M. Quesada-Pérez, J. A. Maroto-Centeno, J. Forcada, R. Hidalgo-Alvarez, *Soft Matter* **2011**, *7*, 10536.
[20] C. G. De Kruif, F. Weinbreck, R. De Vries, R. de Vries, *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 340.

[21] E. Kizilay, A. B. Kayitmazer, P. L. Dubin, *Adv. Colloid Interface Sci.* **2011**, *167*, 24.
[22] A. B. Kayitmazer, A. F. Koksal, E. Kilic Iyilik, *Soft Matter.* **2015**, *11*, 8605.
[23] A. M. Rumyantsev, E. B. Zhulina, O. V. Borisov, *ACS Macro Lett.* **2018**, *7*, 811.
[24] S. Kim, H. V. Sureka, A. B. Kayitmazer, G. Wang, J. W. Swan, B. D. Olsen, *Biomacromolecules* **2020**, *21*, 3026.
[25] C. E. Sing, S. L. Perry, *Soft Matter.* **2020**, *16*, 2885.
[26] S. Yu, X. Xu, C. Yigit, M. Van Der Giet, W. Zidek, J. Jankowski, J. Dzubiella, M. Ballauff, *Soft Matter.* **2015**, *11*, 4630.
[27] X. Xu, Q. Ran, P. Dey, R. Nikam, R. Haag, M. Ballauff, J. Dzubiella, *Biomacromolecules* **2018**, *19*, 409.
[28] Q. D. Ran, X. Xu, P. Dey, S. Yu, Y. Lu, J. Dzubiella, R. Haag, M. Ballauff, *J. Chem. Phys.* **2018**, 149.
[29] Q. Ran, X. Xu, J. Dzubiella, R. Haag, M. Ballauff, *ACS Omega* **2018**, *3*, 9086.
[30] X. Xu, M. Ballauff, *J. Phys. Chem. B* **2019**, *123*, 8222.
[31] K. Achazi, R. Haag, M. Ballauff, J. Dernedde, J. N. Kizhakkedathu, D. Maysinger, G. Multhaup, *Angew. Chem. Int. Ed. Engl.* **2021**, *60*, 3882.
[32] J. J. Walkowiak, M. Ballauff, *Adv. Sci.* **2021**, *8*, 2100661.
[33] J. Bukala, P. Yavvari, J. Walkowiak, M. Ballauff, M. Weinhart, *Biomolecules* **2021**, *11*, 1377.
[34] I. Capila, R. J. Linhardt, *Angew. Chem., Int. Ed.* **2002**, *41*, 390.
[35] D. Hachim, T. E. Whittaker, H. Kim, M. M. Stevens, *J. Controlled Release* **2019**, *313*, 131.
[36] E. Seyrek, P. Dubin, *Adv. Colloid Interface Sci.* **2010**, *158*, 119.
[37] G. P. Szekeres, K. Pagel, Z. Heiner, *Anal. Bioanal. Chem.* **2021**, *414*, 85.
[38] U. Freudenberg, P. Atallah, Y. D. P. Limasale, C. Werner, *Faraday Discuss.* **2019**, *219*, 244.
[39] U. Freudenberg, A. Zieris, K. Chwalek, M. V. Tsurkan, M. F. Maitz, P. Atallah, K. R. Levental, S. A. Eming, C. Werner, *J. Controlled Release* **2015**, *220*, 79.
[40] N. Lohmann, L. Schirmer, P. Atallah, E. Wandel, R. A. Ferrer, C. Werner, J. C. Simon, S. Franz, U. Freudenberg, *Sci. Transl. Med.* **2017**, *9*, eaai9044.
[41] L. Schirmer, P. Atallah, U. Freudenberg, C. Werner, *Adv. Sci.* **2021**, 8.
[42] P. Atallah, L. Schirmer, M. Tsurkan, Y. D. Putra Limasale, R. Zimmermann, C. Werner, U. Freudenberg, *Biomaterials* **2018**, *181*, 227.
[43] Y. D. P. Limasale, P. Atallah, C. Werner, U. Freudenberg, R. Zimmermann, *Adv. Funct. Mater.* **2020**, *30*, 2000068.
[44] S. Kühn, J. Freyse, P. Atallah, J. Rademann, U. Freudenberg, C. Werner, *Biol. Chem.* **2021**, *402*, 1453.
[45] J. J. Walkowiak, M. Ballauff, *Adv. Sci.* **2021**, *8*, 2100661.
[46] J. J. Walkowiak, M. Ballauff, R. Zimmermann, U. Freudenberg, C. Werner, *Biomacromolecules* **2020**, *21*, 4615.
[47] U. Freudenberg, J.-U. Sommer, K. R. Levental, P. B. Welzel, A. Zieris, K. Chwalek, K. Schneider, S. Prokoph, M. Prewitz, R. Dockhorn, C. Werner, *Adv. Funct. Mater.* **2012**, *22*, 1391.
[48] I. Adroher-Benítez, A. Moncho-Jordá, J. Dzubiella, *Langmuir* **2017**, *33*, 4567.
[49] C. Yigit, M. Kanduc, M. Ballauff, J. Dzubiella, *Langmuir* **2017**, *33*, 417.
[50] P. Kosovan, T. Richter, C. Holm, *Macromolecules* **2015**, *48*, 7698.
[51] T. Richter, J. Landsgesell, P. Kosovan, C. Holm, *Desalination* **2017**, *414*, 28.
[52] J. Landsgesell, L. Nová, O. Rud, F. Uhlík, D. Sean, P. Hebbeker, C. Holm, P. Kosovan, *Soft. Matter.* **2019**, *15*, 1155.
[53] O. V. Rud, J. Landsgesell, C. Holm, P. Kosovan, *Desalination* **2021**, *414*, 28.
[54] Q. Ran, X. Xu, P. Dey, S. Yu, Y. Lu, J. Dzubiella, R. Haag, M. Ballauff, *J. Chem. Phys.* **2018**, *149*, 163324.
[55] M. T. Record, C. F. Anderson, T. M. Lohman, *Q Rev. Biophys.* **1978**, *11*, 103.
[56] J. Walkowiak, Y. Lu, M. Gradzielski, S. Zauscher, M. Ballauff, *Macromol. Rapid Commun.* **2020**, 41.
[57] J.-U. Sommer, R. Dockhorn, P. B. Welzel, U. Freudenberg, C. Werner, *Macromolecules* **2011**, *44*, 981.
[58] L. Schirmer, P. Atallah, C. Werner, U. Freudenberg, *Adv. Healthcare Mater.* **2016**, *5*, 3157.
[59] L. Schirmer, U. Freudenberg, C. Werner, *J. Tissue Eng. Regener. Med.* **2014**, *8*, 249.
[60] N. Lohmann, L. Schirmer, P. Atallah, E. Wandel, R. A. Ferrer, C. Werner, J. C. Simon, S. Franz, U. Freudenberg, *Exp. Dermatol.* **2018**, *27*, E18.
[61] W. Zhao, S. A. Mccallum, Z. Xiao, F. Zhang, R. J. Linhardt, *Biosci. Rep.* **2012**, *32*, 71.
[62] L. Liu, P. Chopra, X. Li, K. M. Bouwman, S. M. Tompkins, M. A. Wolfert, R. P. De Vries, G.-J. Boons, *ACS Cent. Sci.* **2021**, *7*, 1009.
[63] X. Xu, Q. Ran, R. Haag, M. Ballauff, J. Dzubiella, *Macromolecules* **2017**, *50*, 4759.