POROVISCOELASTIC MODELING OF PROTEIN HYDROGELS

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ABSTRACT
Many solid food matrices contain high amounts of solvent, typically water. Hence, the structural behavior depends on its biphasic nature. The time dependent mechanical characterization of foods and hydrocoloid-based solids has typically been analyzed following a viscoelastic approach, omitting the effect of the solvent. However, in solvent rich solids the solvent flows internally as it is compressed, for example, which is typically understood using poroelastic theory. A poroelastic approach allows the determination of parameters, such as the Darcy's diffusivity or the intrinsic permeability, that have a physical meaning. The application of poroelasticity to materials has been traditionally limited due to the complex data analysis. Recently, it has been proposed for polymeric hydrogels a novel experimental methodology, based on relaxation after indentation that greatly simplifies the subsequent analysis. This methodology is applied here for the first time to a complex food-like matrix, to heat induced whey protein hydrogels.

Keywords: poroelasticity, finite elements analysis, hydrogel, whey proteins.

1. INTRODUCTION

Solvent rich soft materials, like hydrogels (Chan et al., 2012b) and cells (Trappmann et al., 2012), have recently been characterized using a novel poroelastic relaxation indentation methodology (Hu et al., 2010). The hydrogel, at swelling equilibrium, is indented a small fixed depth using a rigid probe. The relaxation force is measured with time and is subsequently analyzed using correlations obtained from poroelastic finite element analysis. The power of this novel methodology, despite the experimental simplicity, is that several material properties including the shear modulus, diffusion coefficient, average pore size and even the Flory-Huggings interaction parameter, could be obtained by a single experiment (Hu et al., 2011b).
This novel methodology, developed and tested initially for synthetic polymeric systems, has been recently expanded to biomaterials such as gelatin and agar (Strange and Oyen, 2012). We apply it here for the first time to protein hydrogels, and to whey proteins in particular due to their extensive use in research and commercial applications (Mercadé-Prieto et al., 2008). These initial tests were performed in a typical food lab rheometer with good normal force resolution, where the main limitation was that only one cylindrical indenter was available (Fig. 1). Subsequently it is summarized the mechanical analysis of indentation-relaxation tests with a cylindrical punch.

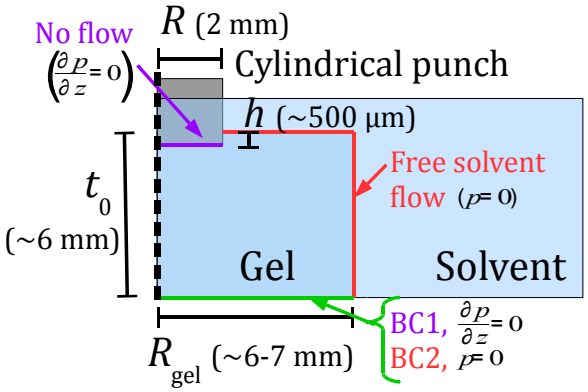


Figure 1. Schematic axisymmetric representation of a swollen protein hydrogel indented with a cylindrical punch. In the finite element analysis it was considered no solvent flow in the area in contact with the indenter (zero solvent flux in the vertical direction,), and free flow (zero pore pressure at the gel surface, $p = 0$) in the non-contact area. For the bottom area, that in contact with the substrate, two boundary conditions are considered: BC1 for no flow, and BC2 for diffusive equilibrium.

2. INDENTATION ANALYSIS

2.1. Elastic loading with a cylindrical punch

The force at an indentation depth h using a flat punch indenter in an elastic (E) sample (Fig. 1) is (Hu et al., 2010):

$$F_E = 8 G_E R h \Pi(R/t_0, \nu) \quad (1)$$

where G_E is the instantaneous shear modulus in a linear elastic material; R is the radius of the indenter, ν is the instantaneous Poisson's ratio, and $\Pi(R/t_0,\nu)$ is a dimensionless function needed to correct substrate effects, important for ratios of the indenter radius to the sample thickness $R/t_0 > 0.1$ (Cao et al., 2009). After loading, the indentation depth h is kept constant, resulting in a constant contact area. Because the indentation step is quick compared to the subsequent relaxation, it is typically treated the hydrogel as incompressible during loading (ν = 0.5).

2.2. Poroelastic (PE) relaxation

A purely elastic hydrogel does not show a force decrease with time t after indentation. A force relaxation in a hydrogel is typically due to the flow of the interstitial fluid through the polymer network – poroelasticity (PE) – and due to the intrinsic viscoelasticity (VE) of the polymer network (Strange et al., 2013). The force relaxation after indentation is first considered for a poroelastic material. Hu et al. (2010) have shown that the normalized relaxation force $F_{PE}(t)$ is only a function of the normalized time τ_{PE}:

$$\frac{F_{PE}(t)-F_{PE}(\infty)}{F_E-F_{PE}(\infty)}=f_{PE}(\tau_{PE}) \tag{2}$$

where $F_{PE}(\infty)$ is the force in the long-time limit. In a cylindrical punch, $\tau_{PE} = Dt/R^2$; where D is Darcy's law diffusivity. It is assumed that both the solvent and the solid matrix are incompressible. The function $f_{PE}(\tau_{PE})$ is found computationally for different indenter shapes and hydrogel configurations, such as different R/t_0 ratios (Hu et al., 2011a). The function $f_{PE}(\tau_{PE})$ is usually fitted to a series of exponential functions such as:

$$f_{PE}(\tau_{PE})=\sum_{i=1}^{N_{PE}} A_i e^{(B_i \tau_i^{C_i})} \tag{3}$$

where A_i, B_i, and C_i are regression constants, and N_{PE} is an integer typically 2 to 3. The extent of relaxation in PE is determined by the Poisson's ratio of the drained hydrogel, ν_d (Hu et al., 2010):

$$\frac{F_{PE}(\infty)}{F_E}=\frac{1}{2(1-\nu_d)} \tag{4}$$

Poroelastic properties of hydrogels are also typically described using the intrinsic permeability k:

$$k=\frac{(1-2\nu_d)D\eta}{2(1-\nu_d)G} \tag{5}$$

2.3. Viscoelastic (VE) relaxation

The viscoelastic relaxation of a material is usually described empirically using a series of exponentials, known as Prony series, of the shear modulus (Cao et al., 2009):

$$G_{VE}(t)=G_E\left(1-\sum_{j=1}^{N_{VE}} g_{VEj}\left(1-e^{(-t/\tau_{VEj})}\right)\right) \tag{6}$$

where g_{VEj} and τ_{VEj} are the relative shear modulus and the relaxation time of the viscoelastic deformation, and N_{VE} is an integer typically between 1 to 4. The normalized relaxation force with time due to viscoelasticity is therefore

$$\frac{F_{VE}(t)}{F_E}=1-\sum_{j=1}^{N_{VE}} g_{VEj}\left(1-e^{(-t/\tau_{VEj})}\right) \tag{7}$$

2.4. Poroviscoelastic (PVE) relaxation

Both relaxation methods, PE and VE, can occur simultaneously resulting in a poroviscoelastic (PVE) relaxation. Both phenomena have been suggested that can be decoupled to compute the overall PVE relaxation (Strange et al., 2013), such as:

$$F_{PVE}(t)=\frac{F_{VE}(t)F_{PE}(t)}{F_E} \tag{8}$$

The extent of relaxation in PVE is expected to be:

$$\frac{F_{PVE}(\infty)}{F_E}=\left(1-\sum_{j=1}^{N_{VE}} g_{VEj}\right)\left(\frac{1}{2(1-\nu_d)}\right) \tag{9}$$

In this paper we implement the above relaxation models to swollen protein hydrogels and discuss their validity.

3. INDENTATION OF PROTEIN HYDROGELS

3.1. Whey protein hydrogels

Heat-induced hydrogels were formed as reported previously (Mercadé-Prieto et al., 2007b), using commercial whey protein isolate powder (BiPro, Davisco, USA). Protein solutions at 15 wt%, with 0.1% sodium azide, were heated inside plastic test tubes for 20 min at 80°C. Gels were stored overnight at 4°C prior being cut in ~6 mm height cylinders, which were then submerged in water solutions at either 0.1, 0.2 or 0.3 M NaCl. The swelling ratio $SR = (m_{sw}/m_0 - 1)$ was calculated from the gels mass difference after swelling equilibrium was achieved (Mercadé-Prieto et al., 2007b).

3.2. Indentation

The swollen hydrogels were placed during testing on a glass petri dish with the corresponding salt solution. Indentation was performed using a rheometer with good normal force resolution, <0.001 N (Malvern Kinexus, UK), with a 2 mm cylindrical indenter. The indenter was placed close to, but not in contact with, the hydrogel surface. Thereafter, the gel was compressed by modifying the gap; the actual indentation depth h was determined a posteriori during the loading analysis.

Gels were allowed to relax for one hour or until no force change was noticeable.

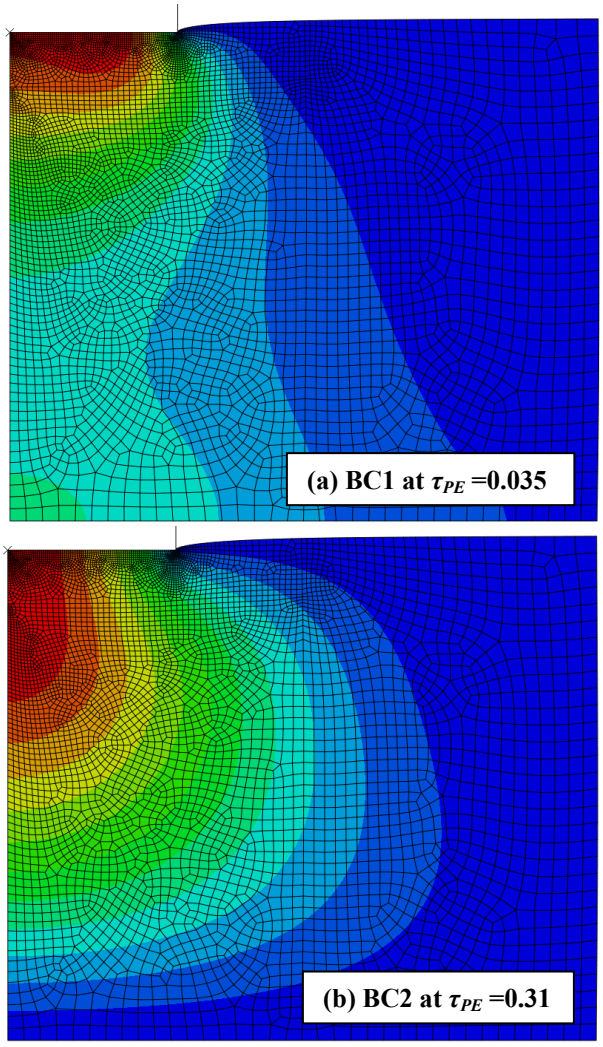


Figure 2. Finite element simulations of the pore pressure inside a swollen hydrogel using $\nu_d = 0$.

4. FINITE ELEMENT ANALYSIS OF INDENTATION RELAXATION

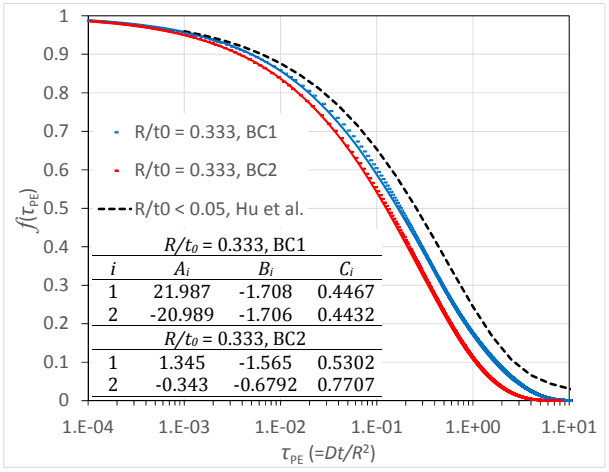
Finite element analysis of the indentation problem shown in Fig. 1 was performed as described by several previous researchers (Hu et al., 2011b; Lin and Hu, 2006; Strange et al., 2013). The main novelty in the present work is that we model small hydrogels using a flat cylindrical indenter, and that we verify PVE eq. 8 using finite elements also for a flat indenter. Although tested gels had some size variations in thickness and diameter, for instance due to the different extent of swelling, the variations where too small to justify using different finite element geometries for each particular gel. For example, the key parameter R/t_0 was 0.32 ± 0.02 (SD) for 0.1 M NaCl gels, 0.32 ± 0.01 at 0.2 M NaCl, and 0.34 ± 0.09 at 0.3 M NaCl, hence an average value of $R/t_0 = 0.333$ was considered in the simulations. In the same way, the diameter of the simulation hydrogel was chosen as $R/R_{gel} = 0.286$. The indentation depth simulated was very small, $h/t_0 < 0.03$, although results where highly insensitive to h up to large values after normalization.

In previous studies, it was assumed no solvent flow through the bottom of the hydrogel, this boundary condition is termed BC1 here (see Fig. 1). In BC1, the pore pressure does increase with time at the gel bottom, shown in Fig. 2(a). This boundary conditions seemed unlikely in our experiments. Hence, simulations were also performed allowing solvent flow in all directions except the area in contact with the rigid indenter (termed model BC2), Fig. 2(b). PE simulations were fitted to eq. 3 with $N_{PE} = 2$; that equation was then used to calculate D from normalized experimental relaxations, as reported in the literature. For PVE analysis, using $N_{VE} = 1$, three parameters where optimized: D, g_{VE1} and τ_{VE1}. The parameter D was optimized using the simplex search method, function *fminsearch* in Matlab, whereas the last two parameters were optimized within fixed bounds, using the function *fminbnd* in Matlab, in order to assure convergence and a physical meaning ($0 < g_{VE1} < 0.25$; $0.5 \text{ s} < \tau_{VE1} < 100$ s). The parameters were optimized one at a time, iteratively, until the mean square error (MSE) did not improve further. All the numerical analysis was performed in Matlab.

5. RELAXATION SIMULATIONS

5.1. Poroelastic simulations

The normalized force for PE given in eq. 2 is very useful to determine the solvent diffusivity D because the shape of $f_{PE}(\tau_{PE})$ does not change with the other material parameters, such as G_E and ν_d, or the extent of indentation h. However, the shape of $f_{PE}(\tau_{PE})$ does depend on size of the indenter in proportion to the hydrogel size, R/t_0, as shown elsewhere for a spherical indenter (Hu et al., 2011a). Figure 3 shows the finite element analysis results for the testing geometry shown in Fig. 1, for the two boundary conditions considered, which is faster compared to a semi-infinite sample, as shown from the results by Hu et al. (2010).



R/t_0 = 0.333, BC1

i	A_i	B_i	C_i
1	21.987	-1.708	0.4467
2	-20.989	-1.706	0.4432

R/t_0 = 0.333, BC2

1	1.345	-1.565	0.5302
2	-0.343	-0.6792	0.7707

Figure 3. Finite element simulations for the PE relaxation for the geometry shown in Fig. 1, and comparison with the results of Hu et al. (2010) for a

semi-infinite hydrogel. Continuous lines are the best fit exponential regressions eq. 3; inset table shows the regression coefficients for $N_{PE} = 2$.

5.2. Poroviscoelastic simulations

The fitting of the experimental relaxation curves in PVE was performed in analogy to that of PE, after normalizing the force as $(F(t) - F(\infty))/(F_E - F(\infty))$. However, if the shape of PE $f_{PE}(\tau_{PE})$ only depends on D for a fixed indenter and sample geometry, in PVE it also depends on the VE parameters g_{VEj} and τ_{VEj}. Here, only one exponential term was needed to model the additional viscoelastic relaxation, i.e. $N_{VE} = 1$. In addition, the PVE relaxation profile also depends on the drained Poisson's ratio ν_d, as it determines the extent of the PE relaxation, see Fig. 4. The drained Poisson's ratio used for PVE fitting was that calculated from the PE analysis; small ν_d modifications due to g_{VE1} during the overall fitting process where not considered for simplicity. The decoupled eq. 8 can describe the PVE relaxation satisfactorily in the experimental range of $0.2 > \nu_d > -0.2$ (Table 1); and the final $F_{PVE}(\infty)/F_E$ using eq. 9 was also well predicted from simulations for the typical parameters considered here, with errors <3%.

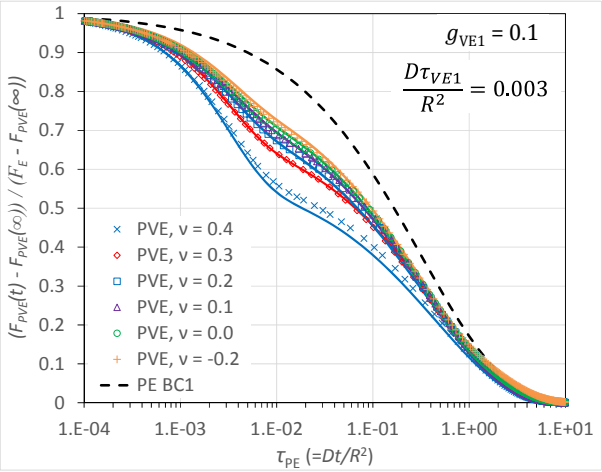


Figure 4. Normalized PVE force at fixed VE conditions and for different drained Poisson's ratio ν_d. Points are results from finite element analysis for the BC1 model, lines are calculated using eq. 8. Note that most experimental results were obtained for $0.2 > \nu_d > -0.2$, hence the relaxation profile does not change significantly.

6. RESULTS

6.1. Hydrogels indentation

Heat induced whey protein gels were soaked in water with three different NaCl concentration to achieve different degrees of equilibrium swelling ratios SR (Table 1). The indentation tests include a loading and a relaxation step at a fixed indentation depth h. From the loading step, the elastic shear modulus G_E was determined assuming a linear-elastic response, eq. 1. The contact point, required to calculate h was determined as shown in Fig. 5. An average corrective value of 1.42 was chosen for $\Pi(R/t_0,\nu)$ in eq. 1 for all experiments. The average G_E found, summarized in Table 1, are comparable to those reported previously for unswollen whey protein gels at similar protein concentrations using a different indentation technique (Özkan et al., 2002).

Table 1. Best fit relaxation parameters for whey protein gels at swelling equilibrium with different salt concentrations (± SD). Different superscripts show significant difference ($p < 0.05$): Latin letters for row comparisons, Greek letters for column comparisons of the same propriety.

	[NaCl] / M	0.1	0.2	0.3
	SR	$0.123^{a} \pm 0.017$	$-0.091^{b} \pm 0.02$	$-0.134^{c} \pm 0.02$
	# tests	34	19	28
	G_E / kPa	$15^{a} \pm 3$	$32^{b} \pm 5$	$36^{c} \pm 7$
	$F(\infty)/F_E$	$0.59^{a} \pm 0.06$	$0.50^{b} \pm 0.07$	$0.44^{c} \pm 0.08$
PE	ν_d	$0.14^{a\alpha} \pm 0.09$	$-0.01^{b\alpha} \pm 0.2$	$-0.2^{c\alpha} \pm 0.3$
PE BC1	D / 10^{-9} m^2 s^{-1}	$8.0^{a\alpha} \pm 3.5$	$6.4^{ab\alpha} \pm 3$	$5.8^{b\alpha} \pm 2$
	Av. MSE	$3.7\text{x}10^{-4\ a\alpha}$	$4.1\text{x}10^{-4\ a\alpha}$	$3.1\text{x}10^{-4\ a\alpha}$
PE BC2	D / 10^{-9} m^2 s^{-1}	$5.0^{a\beta} \pm 3.7$	$4.2^{ab\beta} \pm 2$	$3.9^{b\beta} \pm 1.3$
	Av. MSE	$5.7\text{x}10^{-4\ a\beta}$	$6.7\text{x}10^{-4\ a\alpha}$	$4.8\text{x}10^{-4\ a\beta}$
PVE BC1	D / 10^{-9} m^2 s^{-1}	$6.6^{a\alpha} \pm 3$	$5.3^{ab\alpha\beta} \pm 3$	$5.0^{b\alpha} \pm 1.9$
	g_{VE1}	$0.05^{a\alpha} \pm 0.03$	$0.079^{a\alpha} \pm 0.05$	$0.06^{a\alpha} \pm 0.04$
	τ_{VE1} / s	$8^{a\alpha} \pm 5$	$7.7^{a\alpha} \pm 4$	$6^{a\alpha} \pm 3$
	ν_d	$0.19^{a\alpha\beta} \pm 0.09$	$0.06^{b\alpha} \pm 0.2$	$-0.13^{c\alpha} \pm 0.3$
	Av. MSE	$1.7\text{x}10^{-4\ a\gamma}$	$1.1\text{x}10^{-4\ a\beta}$	$1.5\text{x}10^{-4\ a\gamma}$
PVE BC2	D / 10^{-9} m^2 s^{-1}	$4.0^{a\gamma} \pm 2$	$3.2^{ab\beta} \pm 1.7$	$3.0^{b\gamma} \pm 1.1$
	g_{VE1}	$0.082^{a\beta} \pm 0.04$	$0.12^{b\alpha} \pm 0.06$	$0.10^{ab\beta} \pm 0.04$
	τ_{VE1} / s	$13^{a\beta} \pm 6$	$12^{a\beta} \pm 5$	$11^{a\beta} \pm 4$
	ν_d	$0.21^{a\beta} \pm 0.1$	$0.10^{b\alpha} \pm 0.2$	$-0.08^{c\alpha} \pm 0.3$
	Av. MSE	$1.1\text{x}10^{-4\ a\delta}$	$6.5\text{x}10^{-5\ b\beta}$	$7.8\text{x}10^{-5\ ab\delta}$

6.2. Viscoelastic relaxation analysis

The relaxation of food matrices, such as cheese (Lucey et al., 2003) or yoghurt (Puvanenthiran et al., 2002), has been typically been modeled as an empirical viscoelastic process (Gallegos and Franco, 1999), where the estimated VE parameters do not have a strong physical meaning. A viscoelastic analysis, using eq. 6, was also be applied to the force relaxation of whey protein gels. A value of $N_{VE} = 3$ was required to fit the relaxation data well (Fig. 6); best fit values are shown in Table 2.

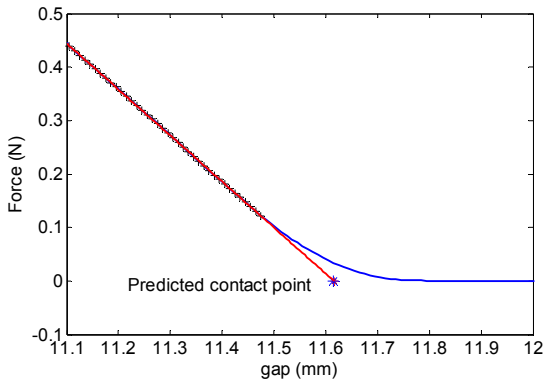


Figure 5. Example of loading step for a hydrogel swollen in 0.3 M NaCl. Red line is the best fit using eq. 1, used to calculate G_E at about 38 kPa here. The predicted contact point, used to determine h, is also shown.

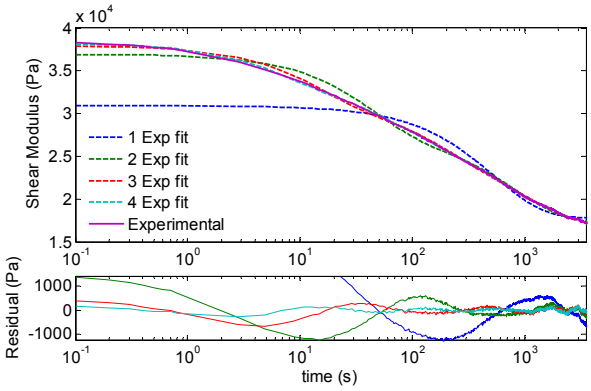


Figure 6. VE analysis with different N_{VE} values for the indentation shown in Fig. 5.

Table 2. Best fit viscoelastic parameters for N_{VE} = 3, estimated using nonlinear regressions of eq. 6.

[NaCl] / M	g_{VE1}	τ_{VE1} / s	g_{VE2}	τ_{VE3} / s	g_{VE3}	τ_{VE3} / s	Av. MSE
0.1	0.12[a] ± 0.03	10[a] ± 3	0.13[a] ± 0.02	107[a] ± 40	0.17[a] ± 0.05	860[a] ± 360	5x10^{-5}
0.2	0.15[b] ± 0.03	10[a] ± 3	0.15[b] ± 0.02	130[ab] ± 45	0.20[a] ± 0.06	1100[ab] ± 400	2x10^{-5}
0.3	0.17[b] ± 0.02	10[a] ± 2.5	0.17[c] ± 0.01	125[b] ± 30	0.25[b] ± 0.07	1000[b] ± 220	2x10^{-5}

6.3. Poroelastic relaxation analysis

The normalized relaxation force for whey protein hydrogels swollen at different salt concentrations was fitted to the PE $f_{PE}(\tau_{PE})$ eq. 3 functions to estimate D. The average D values for the three swelling conditions tested are given in Table 1, with D decreasing slightly with higher salt concentrations, although only the values at 0.1 and 0.3 M NaCl are statistically different. The selection of the model BC1 or BC2 is important as statistically different values are estimated, with BC2 resulting obviously in smaller D values.

The D values estimated are higher than for the water self-diffusion in a comparable whey protein gel (~1.5x10-9 m2 s-1 at 20°C (Colsenet et al., 2005) or in pure water. This result is reasonable because the average pore size of the whey protein hydrogels, estimated at ~20 nm using the PE data, is much larger than the dimension of the water molecule. Consequently, water is expected to flow more by convection rather than by diffusion (Hu et al., 2011b). The intrinsic permeability k of the whey protein gels made is calculated at (0.8–2)x10^{-16} m2, comparable to that reported for agar and acrylamide hydrogels but here for much higher solid concentrations (Oyen, 2014). In fact, the biomaterial more similar to whey protein hydrogels in terms of G_E and k, at similar solid concentrations, would be gelatin (Strange and Oyen, 2012).

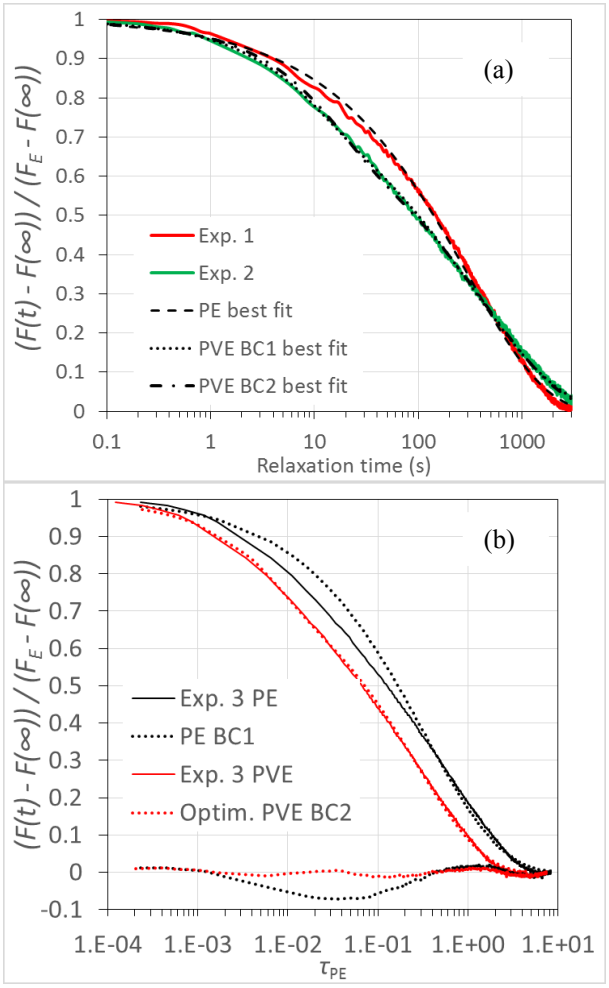


Figure 7. (a) Two representative relaxations of whey protein gels at swelling equilibrium in 0.1 M NaCl. In experiment 1 a PE relaxation fits well (best fit using BC2 model with D = 3.6x10^{-9} m^2 s^{-1}), and little improvement is achieved by considering PVE. Experiment 2 represents the more common tests where fitting is substantially improved by considering PVE relaxation (best fit PVE BC1: D = 4x10^{-9} m^2 s^{-1}, g_{VE1} = 0.074, τ_{VE1} = 10 s, and using PVE BC2: D = 2.4x10^{-9} m^2 s^{-1}, g_{VE1} = 0.11, τ_{VE1} = 16 s). (b) Normalized relaxation

of a hydrogel in 0.3 M NaCl and the best fit using PE BC 1 (black) and PVE BC2 (red) models, D = 9.3x10^{-9} m^2 s^{-1} and D = 4.9x10^{-9} m^2 s^{-1}, g_{VE1} = 0.10, τ_{VE1} = 6 s; respectively. Note that as the estimated D are different, the experimental data (continuous lines) have different τ_{PE} values depending on the model considered.

Experiment 1 in Fig. 7(a) shows one of the few examples (<9% of all tests) where PE could represent well the whole relaxation. The majority of the tests showed a poor agreement with the PE models at 10-100 s, see Exp. 2. This is clearly seen in another test shown in Fig. 7(b), where the residuals of the PE fit show a systematic over prediction at τ_{PE} ~0.01-0.1. Similar relaxation profiles have been reported in the literature, such as for chemically crosslinked polyethylene glycolmethacrylate (PEGMA) (Chan et al., 2012a), and have been explained by considering an additional viscoelastic relaxation, therefore a PVE model.

6.4. Poroviscoelastic relaxation analysis

Two examples of PVE regressions are shown in Fig. 7. The average PVE parameters found are summarized in Table 1. The addition of a viscoelastic relaxation results in lower D estimates, where again are lower using the BC2 model compared to BC1. On absolute terms, the D values estimated are similar to the self-diffusivity of free water (~2x10^{-9} m^2 s^{-1}), particularly using the BC2 model. The viscoelastic parameters g_{VE1} and τ_{VE1} calculated were not affected by the swelling degree. We note that the overall g_{VE1} and τ_{VE1} obtained from PVE are comparable to those found using a VE analysis only (g_{VE1} ~0.15 and τ_{VE1} = 10 s, Table 2). The use of model BC2 was hardly justified for PE analysis as the average MSE was indeed worse compared to BC1. However, for PVE analysis, BC2 fits better the experimental data, validating its use for comparison, and suggesting that solvent equilibration could be possible from the bottom of the hydrogels. The quality of the BC2 model fit, as shown by the average MSE values, is comparable to that of a pure VE analysis with N_{VE} = 3 (Table 2), but with half the number of adjustable parameters. The selection of the model did statistically influence the estimated g_{VE1} and τ_{VE1}, which is likely due to the smaller D estimated with model BC2. We observed a strong a correlation between the estimated D and the VE parameters, not shown. In any case, the values of τ_{VE1} at 6-13 s are comparable to those found for PEGMA hydrogels in an analogous PVE analysis, at 6-20 s (Chan et al., 2012a).

7. DISCUSSION

It was unexpected that the main difference between the whey protein hydrogels soaked at different salt concentrations, other than G_E which is directly related to the extent of swelling, was $F(\infty)/F_E$ and therefore ν_d (Table 1). In addition, it appears that the calculated D correlate with $F(\infty)/F_E$: tests with high $F(\infty)/F_E$ ratios show comparatively high D values (Fig. 8), as well as smaller g_{VE1} and τ_{VE1} for PVE fits.

Comparable small and negative ν_d values have been reported in the literature in agar-gelatin composite hydrogels when the hydrogels were more agar rich, with $0 > \nu_d > -1$ (Strange and Oyen, 2012); and with ν_d ~ -0.7 for pure agar hydrogels calculated with a PVE analysis (Strange et al., 2013). The reasons are unclear, and further tests are required to verify this finding. However, we note that if there were an additional VE relaxation with τ_{VE2} ~200 s, it would be impossible to discern from a PE relaxation in the current experimental setup.

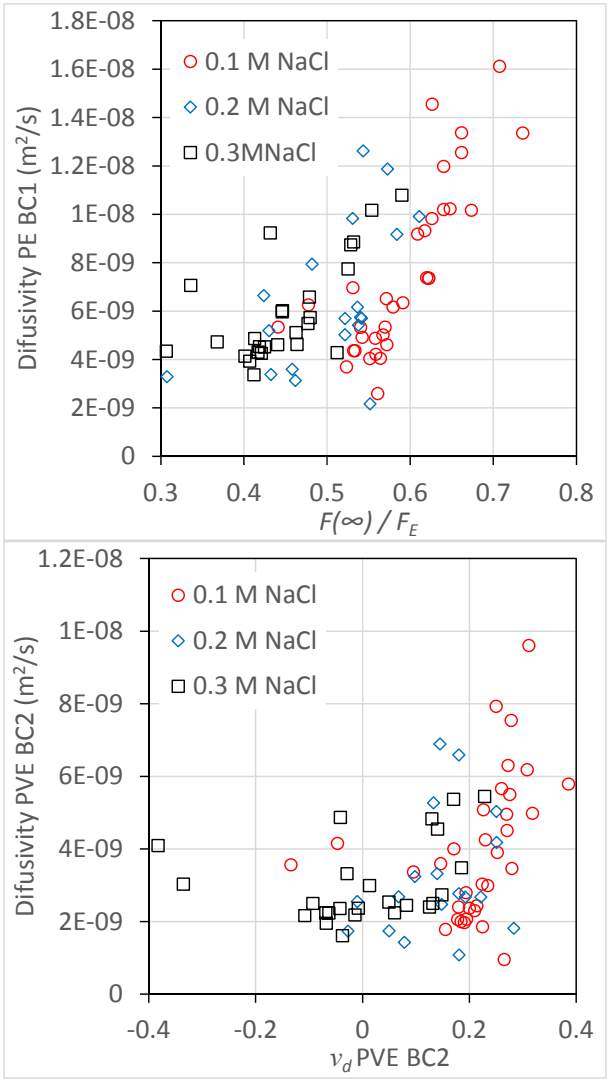


Figure 8. Diffusivites calculated assuming (a) poroelastic relaxation with no solvent flow from the bottom of the gel (BC1), and (b) poroviscoelastic relaxation with solvent flow from the bottom (BC2). Note the different y-axis scale.

One of the advantages of indentation testing to characterize hydrogels is that VE relaxation is independent of the indenter size R, whereas PE relaxation is strongly dependent on R (Wang et al., 2014). Hence, it is theoretically possible to perform experiments with different size indenters to elucidate the validity of the g_{VE1} and τ_{VE1} estimated here.

Unfortunately, only one indenter size was currently available in the rheometer used. In order to decouple more both relaxations in time, there are two options: (*i*) increase the indenter size to $R > 1$ cm, larger than the current size of the gels R_{gel}. Keeping the same indenter – hydrogel proportions than used here, the protein hydrogels should be $R_{gel} > 3$ cm and $t_0 > 3$ cm, which are highly impractical and expensive to make for routine testing. If the hydrogel dimensions were not so large for $R > 1$ cm, then the PE relaxation will occur much faster due to the closer boundary surfaces (Hu, Chan, et al., 2011), canceling the time decoupling effect of increasing R. Option (*ii*) would be to reduce significantly the indenter size, as in nanoindentation (Oyen, 2015). With very small indenters, the PE relaxation would occur before the VE one; for the conditions used here, it is predicted $R < 30$ μm to clearly see both relaxations. $R < 100$ μm should be used to check if there is a VE relaxation with τ_{VE2} ~200 s as discussed above. Different indentation techniques, such as AFM where PE relaxation occurs in the order of seconds or faster (Kalcioglu et al., 2012), would have to be used for such experiments.

The poroelastic indentation methodology has been further used in polymer hydrogels to obtain reasonable estimates of the Flory-Huggings interaction parameter χ (Hu et al., 2011b). It would be very convenient to be able to perform such analysis, despite its many limitations and simplifications, to other more complex food based systems, such as the protein hydrogels considered here. It is known from swelling experiments that χ for whey protein gels is ~0.5 (ideal or Θ conditions) (Mercadé-Prieto et al., 2007a). Yet, according to the figures provided by Hu et al. (2011b), such reasonable χ can only be obtained from indentation data for $0.25 < \nu_d < 0.3$, much higher than determined here. Previous studies on whey protein gels highlight that the extent of relaxation $F(\infty)/F_E$ after indentation is yet poorly understood, as it depends significantly on the gelation conditions for instance (Shim and Mulvaney, 2001). Future work is needed to validate ν_d with a different methodology, to check if a VE relaxation overlaps in time with the PE relaxation, or if a different theoretical framework is required.

8. CONCLUSIONS

We have applied the experimental methodology of indentation-relaxation developed to characterize biomaterials to protein hydrogels. A poroelastic (PE) analysis, where the relaxation is sorely caused by the internal flow of solvent in the hydrogel, provides reasonably good fits of the experimental data, yet it is significantly improved (as extra regression parameters are used) if a small viscoelastic relaxation at short times is considered. The calculated diffusivity D and intrinsic permeabilities k are reasonable and comparable to other hydrogels in the literature. Extensive indentation experiments were conducted with whey protein gels in swelling equilibrium with solutions with different salt concentrations, thus at different swelling ratios. The estimated D increased slightly in more swollen gels, as expected, but it was unexpected that the drained Poisson's ratio ν_d decreased at high salt concentrations to values equal to zero or even negative, which requires further investigation. The proposed PE analysis considers for the first time that solvent equilibrium can also occur from the bottom of the hydrogel (BC2 model), reasonable from an experimental point of view, which improves the PVE relaxation fits and yields D values slightly higher than that of the self-diffusion of free water.

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