A low molecular weight hydrogel which exhibits electroosmotic flow and its use as a bioreactor and for electrochromatography of neutral species†

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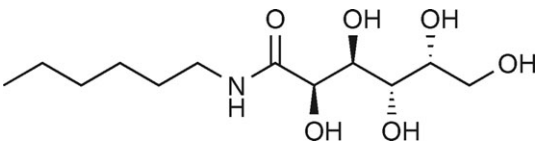
A low molecular weight hydrogel which exhibits electroosmotic flow is described, and its use for separation and biocatalytic applications that require passage of a solvent stream through the gel is demonstrated.

Small molecules which self-assemble through non-covalent bonds to form gels in organic solvents and water continue to attract attention, and their potential applications continue to increase.[1] They are now potentially useful in many fields, some of which require mass transport and interaction with the environment such as sensors,[2] drug delivery,[3] and chemical[4] and biochemical[5] catalysis. For these applications a means to introduce a stream of solvent carrying reactants and products is needed. Needless to say, a pressure driving force would result in deformation or even total destruction of these soft gels. Our group has recently introduced the use of such a low molecular weight organogel (LMOG) as a matrix for electrophoresis.[6] Amino acids and peptides were separated in both slab and capillary form. The most important feature of these gels is that they can be made to revert reversibly to solutions by elevating the temperature or by exertion of shear force which allows easy separation of analytes, reactants and products from the matrix and even direct mass spectrometry analysis. One drawback is that because the gel inhibits electroosmosis as noted in our earlier work, only charged species can be separated, as the solvent itself is not introduced into the capillary. In this work we show that it is possible to promote electroosmotically driven solvent flow through the capillary without destroying the gel. Moreover, electroosmosis can be promoted even in flat slab form where the external walls cannot induce electroosmosis.

As a continuation of our prior work, we searched for a low molecular weight hydrogel in order to be able to work with biomolecules. Very few small molecule hydrogelators are applicable to electrophoresis as most are charged and would thus move in the electric field and destroy the gel. For example, an aqueous *N*-hexadecyl lactosylamine gel was destroyed when we applied an electric field. As a potential matrix we selected a class of hydrogelators consisting of *N*-octyl-hexonamides originally introduced by Furhop *et al.*[7] In order to form organogels for electrophoresis there must be sufficient time to cast the hot solutions into the slabs or capillaries before gelation takes place. Octyl-galactonamide gels at very high temperature (close to 100 °C) which does not allow for formation of gel slabs before cooling. In order to use the galactonamide gel, the alkyl chain was shortened to increase solubility and lower the gelation temperature. The structure of the hexyl-galactonamide gelator which we used is shown in Scheme 1.



Scheme 1 *N*-Hexyl-galactonamide gelator.

While performing separations in slab hydrogels, it was observed that even uncharged molecules were mobile within the gel and were fractionated. Thus there appeared to be flow of the bulk fluid in the gel, a phenomenon that could only be caused by electroosmosis. As the Teflon walls of the planar cell were certainly not charged, the only possible remaining source of charge was the gel itself. Electroosmosis is usually observed in silica capillaries, the walls of which have a negative charge. Electroosmosis can be altered by modifying the capillary walls with charged or uncharged groups or by coating the walls with polymers to block the charged silanol groups. Our previous separation work[6] relied on electrophoresis in order to introduce charged analytes into the bulk *bis*(dodecylamido)-cyclohexane organogel as the gel completely inhibited electroosmosis.

As no clear ionogenic group is present in the gelator, the gelation process must provide the environment necessary for the gel to acquire charge. Of all the hydroxyl groups present, the one adjacent to the amide is the most acidic due to inductive effects, yet not sufficiently so to explain the electroosmosis. It has been observed in gels, polymers, and proteins that hydrogen bonding lowers the pK_a of acidic groups by stabilizing the basic form.[8] In the case of the galactonamide gel, the α-hydroxyl is surrounded not only by the other hydroxyls in the same molecule but also all the other adjacent molecules in the bundle of gel fibers. This great potential for hydrogen bonding stabilization of the resulting base allows for the deprotonation of the gelator molecules. The neutral molecule mesityl oxide was used as a probe for electroosmosis. Its electroosmotic mobility was observed at different pH values. The results are depicted in Fig. 1. Electroosmosis was observable down to pH 4. A parallel can be drawn between these gel fibers and the walls of a silica column whose silicic acid precursor has a pK_a of about 9.6[9] and exhibits electroosmosis down to about pH 2. In both cases, the very same functional group exhibits a different proton dissociation constant depending on its close environment, nearby fibrillar charge, and the availability of

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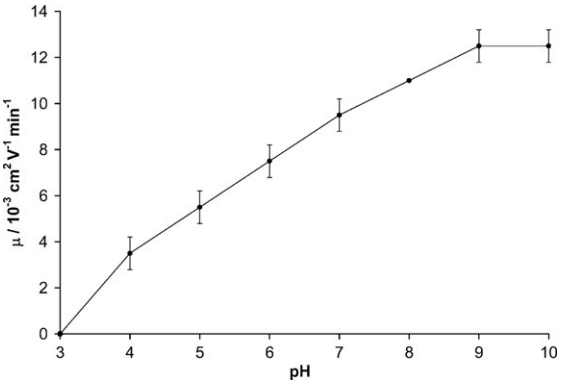

Fig. 1 Effect of pH on mobility of mesityl oxide at 200 V, 7 mg ml^{-1} gelator. Bars represent standard deviation of three gels.

stabilizing hydrogen bonding. This results in a spread of the increase of the fibrillar charge over several pH units.

The effect of changing the concentration of the gelator on the mobility of mesityl oxide was also measured. The results at pH 7 are shown in Fig. 2. A maximum is observed at 9 mg ml^{-1}. We believe that there is a trade-off between two competing factors. On the one hand, the more gelator there is, the more charge accumulates on the fibrillar structure per unit volume and the electroosmotic mobility should increase. On the other hand, as the gel becomes more dense the viscosity increases and the pore volume decreases. Therefore the mobility decreases.[6] The result is the observed local maximum.

In order to rule out the possibility of the electroosmosis being caused by ions from the buffer being adsorbed onto the gel surface, the mobility of mesityl oxide was measured in a variety of buffers. The initial experiments were performed using a wide pH range citrate–phosphate buffer. Changing the concentration of the buffer (at the same pH) did not affect the mobility. Changing to a trisamine based buffer also did not change the mobility. Were the electroosmosis to arise from adsorbed ions, an increase in buffer concentration should have increased the amount of adsorbed ions and hence the electroosmotic flow (EOF). If negative phosphate ions were what caused the electroosmotic flow, replacing them with positive tris ions should have either eliminated the flow or perhaps reversed its direction if the tris ions were adsorbed onto the gel. Details of these experiments are available in the electronic supplementary information (ESI).†

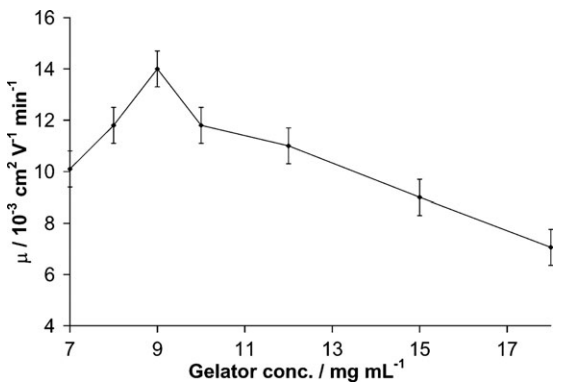

Fig. 2 Effect of gelator concentration on mobility of mesityl oxide at 200 V, pH 7. Bars represent standard deviation of three gels (in each gel at least five distance points were taken as a function of time).

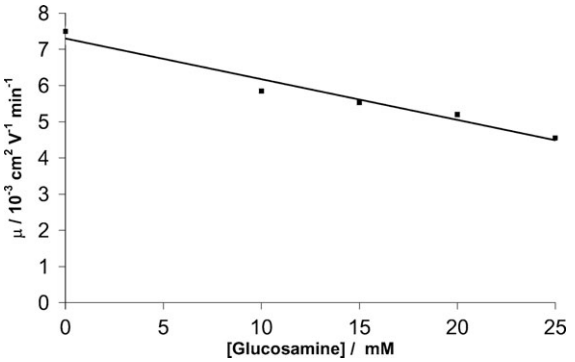

Fig. 3 Effect of the concentration of glucosamine in the buffer on the mobility of mesityl oxide at 200 V, pH 6, 7 mg ml^{-1} gelator.

When glucosamine, a compound which should be easily incorporated into the gel, was added to the buffer, the mobility did decrease as the positive amine partially negated the negative charge of the gel. The change was linear with the concentration of glucosamine as seen in Fig. 3. Ethanolamine, which does not have a strong affinity to the gel like glucosamine, did not cause a decrease in electroosmosis. This demonstrates a method of controlling the EOF. Another way of doing this is to add a polymer such as polyethylene glycol to the buffer.

One potential use of the EOF is for the separation of neutral compounds. Dichlorophenols (DCPs) were chosen as model compounds to demonstrate this application. 2,3-, 2,6- and 3,5-DCP were separated at pH 4. The retention times are shown in Table 1. 2,3-, 2,4- and 2,5-DCP were not resolved. 3,4- and 3,5-DCP were not resolved either. The chlorine location appears to play a key role in the separation, as only compounds with different numbers of chlorine atoms adjacent to the hydroxyl were separated. The hydroxyl group should show affinity to the gel due to hydrogen bonding. 2,6-DCP, which has two chlorines adjacent to the hydroxyl, elutes first, suggesting that the chlorine interferes with the attraction to the gel. 2,3-DCP, which has one chlorine adjacent to the hydroxyl, eluted second and 3,5-DCP with no chlorines adjacent to the hydroxyl eluted last. This can only result from a chromatographic separation based on the differing interactions with the gel. Were charge to play a role, the order of the DCPs would have been reversed, as they would be anions migrating electrophoretically against the EOF and therefore the most acidic would elute last. Since the more acidic compounds eluted first, it must be concluded that charge was not a factor, which is not surprising at a pH much below the pK_a of the compounds.

Another potential use for the EOF promoting gel is in enzyme immobilization, be it for sensing or packed bed bioreactors. Despite the importance of enzymatic reactions in contemporary materials and the continual search for innovative enzyme immobilization systems, the first and only report of enzyme encapsulation in an LMOG was very

Table 1 Retention times and properties of dichlorophenols

	Retention time/min	pK_a
2,6-Dichlorophenol	55.7	7.02
2,3-Dichlorophenol	57.9	7.44
3,5-Dichlorophenol	60.7	8.04

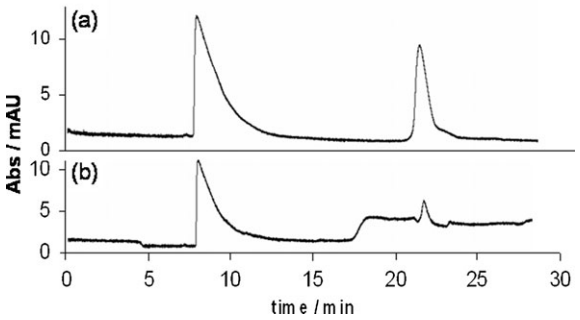

Fig. 4 Electropherograms of pyrogallol (23 min) with *N*-benzyl-1-(1-naphthyl)-ethylamine as internal standard (8 min) in (a) a plain gel capillary; and (b) a capillary with added HRP and hydrogen peroxide. Capillary effective length 42 cm; driving force 20 kV.

recent.[10] Since the presence of electroosmosis in the gel allows the flow of uncharged compounds through the capillary, it is possible to use the gel-filled capillary as a packed bed reactor. To date non-destructive accessibility to the gel interior could be attained only by diffusion or electrophoresis. The first is a slow process and the latter is limited to charged species. This communication expands the possibilities of enzyme immobilization and paves the way for LMOG applications requiring fast transport of analytes, reactants or products between the interior of LMOG gels and their surroundings.

In order to demonstrate this idea, horseradish peroxidase (HRP) and hydrogen peroxide were added to the gel, and pyrogallol was injected into the capillary along with a reference species *N*-benzyl-1-(1-naphthyl)-ethylamine. The conversion was calculated from the ratio of the pyrogallol peak to the reference peak in relation to the ratio in a blank run with no enzyme. All runs were carried out at pH 5 since at higher pH the stronger EOF causes some instability in the gel. A run at 20 kV is shown in Fig. 4. The top window shows a run without HRP. The first peak is the *N*-benzyl-1-(1-naphthyl)-ethylamine internal standard, and the second is pyrogallol which amounts to 46% of the internal standard peak. In the lower window is the run with HRP in which the pyrogallol peak has been reduced to 8% of the internal standard peak. This amounts to an 83% conversion. The oxidation of pyrogallol to purpurogallin is a multi-step reaction with several intermediates.[11] Because the reaction is taking place under electrophoretic conditions, the products are formed at different locations along the capillary and therefore do not emerge as chromatographic peaks, rather they are smeared through the capillary as seen by the rise in baseline starting a few minutes before the pyrogallol peak. No reaction (or decrease of the pyrogallol peak) was observed for thermally denatured HRP.

The oxidation of pyrogallol is first order with respect to each of pyrogallol, peroxidase and peroxide at low substrate concentrations.[12] The effective residence time of pyrogallol in the reactor was changed by varying the voltage and/or the capillary length, keeping all other parameters constant. The data fit the expected logarithmic relationship for first order reactions ($R^2 = 0.991$). The observed rate constant for the enzyme and peroxide concentrations used is 0.0677 min^{-1}. Fig. 2 of the ESI† shows the characteristic logarithmic conversion–retention time dependence. We did not attempt to increase the substrate concentration to observe a Michaelis–Menten dependence.

In conclusion, LMOGs based on octyl hexonamides were found to exhibit electroosmosis, even in slab gels. This is the first demonstration of electroosmosis in an LMOG. This phenomenon is both novel and unexpected. Intuitively, electroosmosis could not be driven by uncharged moieties. However, *post priori*, the occurrence of EOF can be readily explained by the supramolecular structure of LMOGs which imposes environmental constraints which spread the proton dissociation susceptibility of the very same gelator functionality (*i.e.*, the α-hydroxyl) over a wide pH range when it is exposed to different solid environments. This structure can similarly explain the somewhat surprising existence of enzymatic activity within the gel. The gelator, which like most LMOG gelators is a surface active agent having distinct hydrophobic and hydrophilic parts, should have denatured the enzyme. However, this does not happen due to the supramolecular structure which binds and hinders the mobility of the surfactant gelator and thus interferes with its denaturing activity.

The gelator, hexyl-galactonamide was tailored to meet the needs of two model applications. First it was used as a stationary phase for electrochromatography. Dichlorophenols were separated based on the different interactions with the gel resulting from the location of the chloride groups relative to the hydroxyl. The gelator was further used as the solid phase of a packed bed bioreactor hosting HRP inside a fused silica capillary. Pyrogallol was oxidized by hydrogen peroxide catalyzed by the immobilized HRP. The unique properties of LMOGs and especially their thixotropy, reversibility and porosity combined with the accessibility due to electroosmosis thus provide an environment suitable for many analytical applications, two of which were demonstrated here.

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Notes and references

1. M. George and R. G. Weiss, *Acc. Chem. Res.*, 2006, **39**, 489; N. M. Sangeetha and U. Maitra, *Chem. Soc. Rev.*, 2005, **34**, 821; M. de Loos, B. L. Feringa and J. van Esch, *Eur. J. Org. Chem.*, 2005, 3615.
2. P. Mukhopadhyay, Y. Iwashita, M. Shirakawa, S. Kawano, N. Fujita and S. Shinkai, *Angew. Chem., Int. Ed.*, 2006, **45**, 1592.
3. A. Friggeri, B. L. Feringa and J. Van Esch, *J. Controlled Release*, 2004, **97**, 241.
4. Q. Wang, Z. Yang, X. Zhang, X. Xiao, C. K. Chang and B. Xu, *Angew. Chem., Int. Ed.*, 2007, **46**, 4285.
5. S. Kiyonaka, K. Sada, I. Yoshimura, S. Shinkai, N. Kato and I. Hamachi, *Nat. Mater.*, 2004, **3**, 58.
6. S. Mizrahi, J. Gun, Z. G. Kipervaser and O. Lev, *Anal. Chem.*, 2004, **76**, 5399.
7. J. H. Furhop, P. Schnieder, E. Boekema and W. Helfrich, *J. Am. Chem. Soc.*, 1988, **110**, 2861.
8. M. A. Porter, J. R. Hall, J. C. Locke, J. H. Jensen and P. A. Molina, *Proteins*, 2006, **63**, 621.
9. *CRC Handbook of Chemistry and Physics*, ed. R. C. West and M. J. Astle, CRC Press, Boca Raton, FL, 63rd edn, 1982.
10. Q. Wang, Z. Yang, L. Wang, M. Ma and B. Xu, *Chem. Commun.*, 2007, 1032.
11. K. Miyazaki, S. Arai, T. Iwamoto, T. Masaru and A. Tomoda, *Tohoku J. Exp. Med.*, 2004, **203**, 319.
12. M. K. Mahanti, *Afinidad*, 1991, **48**, 59.

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