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Microstructure-driven self-assembly and rheological properties of multi-responsive soft microgel suspensions

Eva Dieuzy^{1,2,3} ‡, Garbine Aguirre^{1,3} ‡, Stéphane Auguste,^{3,4} Kamel Chougrani,⁵ Valérie Alard,⁵ Laurent Billon, ^{1,2,3} * Christophe Derail ^{1,2} *

¹Universite de Pau et Pays de l'Adour, E2S UPPA, CNRS, Institut des Sciences Analytiques & de PhysicoChimie pour l'Environnement & les Matériaux, UMR5254, 64000, PAU, France

²LERAM, LabCom UPPA/URGO, Hélioparc, 2 avenue Angot, 64053 Pau

³Bio-inspired Materials group: Functionalities & Self-assembly, E2S UPPA, Hélioparc, 2 avenue Angot, 64053, Pau.

⁴ URGO RECHERCHE INNOVATION ET DEVELOPPEMENT; 42 Rue de Longvic, 21300 Chenôve, France

⁵LVMH Recherche Parfums et Cosmétiques ; 185 Av. De Verdun, St Jean de Braye F-45804, France.

Abstract

Hypotheses

The deformation and swelling ability of microgels is influenced by the crosslinking distribution. Varying microgels microstructure is expected to obtain suspensions with different flow behavior and thereby, different rheological properties.

Experiments

Different multi-responsive microgels were synthesized using two different crosslinkers and varying their amounts: N,N-methylene bis-acrylamide (MBA) and oligo(ethylene glycol) diacrylate (OEGDA). The rheological results were obtained by zero-shear viscosity and long-time creep measurements on concentrated microgel suspensions Microgel microstructure was analyzed by ¹H nuclear magnetic resonance transverse relaxation measurements.

Findings

At a constant crosslinking rate, we show that the viscosity of OEGDA-crosslinked microgels diverges at a higher concentration than MBA ones, suggesting a looser shell and less restricted dangling chains at the periphery for the later. By scaling with the effective volume fraction, the viscosity curves of the different microgel suspensions reduce into a single curve and closely follow hard sphere models up to $\phi_{eff} < 0.45$. The results from creep tests revealed a much higher yield stress for MBA-crosslinked microgels, strengthening the hypothesis of a looser shell for the later. Finally, transverse relaxation (T₂) NMR measurements demonstrated that, although all microgels exhibit a core-shell microstructure, MBA samples present a less crosslinked shell corroborating with the rheological results.

Keywords: microgels, stimuli-responsive, microstructure, rheology, yield stress

Introduction

Microgels are colloidal particles that are composed of cross-linked polymeric networks swollen in a good solvent.¹ Thanks to their high swelling ability, microgels are attractive for food, cosmetic and pharmaceutical applications, in which they are commonly used as texture modifiers²⁻⁴ or bioactive delivery systems.^{5,6} In contrast to hard sphere systems, microgels are deformable particles that present a hairy surface of swollen dangling chains. The soft structure strongly affects the flow behavior of suspensions, especially when particles enter in close contact. It is well-established that, above a critical volume fraction, hard particles form a dense network that exhibits solid-like properties, including elasticity and yield stress.⁷ This critical volume fraction corresponds to the random close packing, noted RCP, ($\phi_{RCP} \approx 0.64$) for monodispersed hard spheres.⁸ However, it is greatly influenced by the particle characteristics such as size, shape or polydispersity.⁹⁻¹¹ Microgels represent a much richer and more complex system than hard spheres due to their softness and to their structure. At high concentration, microgels may undergo deformation, interpenetration and deswelling which enable the suspension to be concentrated at higher volume fraction than the physical limit of RCP.¹²⁻¹⁵ Crosslinking density of microgels, which directly impacts the deformation and deswelling capacity, represents an important lever to tune the rheological characteristics such as viscosity, modulus and yield stress.^{16,17}

Furthermore, the possibility to synthesize stimuli-responsive microgels, such as thermo- and pHsensitive ones, have brought considerable interest both in academia and industry. Poly(Nisopropylacrylamide)-based microgels are considered as model thermo-responsive systems and have been widely studied in view of their high encapsulation efficiency. The Lower Critical Solution Temperature (LSCT) of PNiPAM chains at around 32°C induces a sharp volume transition of microgels at about the same temperature.¹⁸ Such thermo-responsive behavior leads to interesting rheological features, such as the tunability of the effective volume fraction by the temperature for a constant particle concentration. More interestingly, at the swollen-to-collapse transition, the suspension evolves from a repulsive to an attractive system due to the change of solvent quality, resulting in the disappearance of steric forces for example.^{19,20} While the influence of crosslinking density on the flow behavior of microgels has been extensively investigated, rheological studies on the impact of the microgel microstructure are scarce. Yet, one can expect that the microstructure of microgels in terms of crosslinking distribution probably affects as much the flow behavior since it also influences the swelling ability. Regarding the microstructure of the microgels, the general concept for microgels prepared by batch emulsion/precipitation polymerization is a core-shell model in which core and shell have different structures and crosslinking degrees.^{21,22} The reason of this heterogeneous crosslinking density distribution is the faster reaction rate of crosslinkers than that of main monomers.²³ Up to now, the heterogeneous microgel structure has been studied using different techniques such as static light scattering,²⁴ small-angle neutron scattering (SANS),^{14,25,26} and ¹H-nuclear magnetic resonance (¹H NMR) transverse relaxation measurements.^{21,27,28}

Recently, Balaceanu and coworkers reported Flory temperature-induced volume transition theory for homopolymer microgels with bimodal heterogeneous morphology. ²⁸⁻²⁹ In the same way, Pikabea *et al.* generalized the theory for thermo- and pH-responsive nanogels with different bimodal heterogeneous morphologies.³⁰ In these works, the quantitative characterization of coreshell morphology such as the number of chains between two crosslinking points into the core and shell and the relative volume of the core, was possible thanks to some microscopic and

thermodynamic constraints imposed by ¹H-transverse relaxation NMR and Flory equation of state.

The novelty of this contribution comes from the study of the effect of different crosslinkers on the thermo- and pH-responsive microgels rheological behavior together with the understanding of their inner structure effect on their rheological properties. Although up to now, multiresponsive microgels based on PNIPAM are the most studied ones in terms of thermoresponsiveness, the use of poly(oligoethylene glycol) methacrylates (POEGMA) could be a better alternative thanks to their biocompatibility in the design of multi-responsive microgels.^{32,33} In this sense, biocompatible oligo(ethylene glycol)-based microgels were synthesized by precipitation polymerization using as reference the procedure reported by Boularas *et al.*²². They suggested the different conversion rates of the crosslinkers should induce different complex microstructures, such as homogeneous and core-shell ones. During the solvent evaporation, the suspensions successively evolve from a fluid to a soft paste, and finally to a viscoelastic film thanks to the self-assembly of microgels.^{6, 31} In a first step, the present study aimed at using rheology as a tool to get insight on the microgel microstructure and formulate hypotheses on the difference in fuzziness caused by the crosslinker type and rate. Indeed, the interactions between microgels during flow in a crowded state can reveal understanding of the microstructure of the particle itself. In a second step, the objective was to confirm these hypotheses using proton transverse relaxation NMR measurements. The understanding of the particle microstructure will be extremely helpful at a later stage to discuss about the mechanical properties of films formed on the self-assembly of microgels. The study of the film properties will be the central subject of a coming publication. In this work, the influence of the crosslinking density and crosslinking distribution was investigated by varying respectively the crosslinker rate (2 mol.% versus 8 mol.%) and the crosslinker type (OEGDA *versus* MBA). Although the impact of crosslinking density is well-described in the literature, the motivation to study a higher crosslinking rate was to validate the suitability of the rheological tests in revealing structural differences for a known microstructure. Zero-shear viscosity and creep tests were performed to investigate the flow and yielding behavior of these four microgel suspensions. Since the investigation of microstructure is the main objective, the study focused on measurements under the volume phase transition, that is to say when microgels are swollen and dominated by repulsive electro-steric forces. To confirm the macroscopic hypotheses based on the rheology study, the microgels microstructure was determined by proton transverse relaxation NMR measurements together with the application of Flory-Rehner theory to our microgels.

Materials & Methods

Materials. Di(ethylene glycol) methyl ether methacrylate (MEO₂MA, 95%), oligo(ethylene glycol) methyl ether methacrylate (OEGMA, terminated by 8 EG units with Mn=475 g·mol^(-1)), methacrylic acid (MAA), poly(ethylene glycol) diacrylate (OEGDA, Mn=250 g·mol^(-1)), N,N-methylenebisacrylamide (MBA) and potassium persulfate (KPS) were purchased from Sigma Aldrich and used as received. Deuterium oxide (D₂O), supplied by Eurisotop, was used for NMR characterization. Purified water from a Millipore Milli-Q system was used.

Microgel synthesis. The precipitation polymerization of microgels is carried out in a 2 L jacketed glass reactor by following the procedure previously described by Boularas *et al.*^{35,36} Briefly, MEO₂MA (92.6 mmol), OEGMA (10.3 mmol) and varying amounts of crosslinker,

either OEGDA or MBA, are dissolved in 930 g of water. The crosslinker ratios are set at 2 mol.%, and 8 mol.% *versus* the total vinylic molecules, respectively corresponding to 2.12 mmol, and 9.42 mmol. The mixture is introduced in the 2 L reactor and stirring is set at 150 rpm. The reactor is purged with nitrogen for 45 min to remove oxygen at room temperature. MAA (5.41 mmol) is dissolved in 30 g of water and added to the reactor. The mixture is heated to 70°C. Finally, KPS (0.958 mmol) is dissolved in 40 g of water and injected into the reactor to start the reaction. The reaction is kept at 70°C during 6 hours. At the end of synthesis, microgels are purified by 3 centrifugation cycles (20,000 rpm, 20 min). The microgel suspensions are abbreviated by the molar ratio and the crosslinker type. The four systems studied are then called: 2-MBA, 2-OEGDA, 8-MBA and 8-OEGDA.

Methods. Hydrodynamic diameters (D_h) were measured by Dynamic Light Scattering (DLS) on a Zetasizer Nano (Malvern), at an angle of 173° and a laser wavelength of 633 nm. Microgel dispersions were diluted to a concentration of c = 1 mg.mL⁻¹. Temperature ramps were realized from 20°C to 60°C, with 3 measurements each 2.5°C. A stabilization time of 10 minutes was used between each set of measurements.

The relative viscosity of dilute suspensions was investigated with a homemade computercontrolled capillary viscosimeter developed by Rodriguez *et al.*³⁷ Five to six concentrations of dilute suspensions were measured for each samples.

The rheological measurements of concentrated suspensions were performed on a stresscontrolled Bolhin Gemini (Malvern) using a concentric cylinder geometry (inner and outer diameters: 25mm and 27.5 mm). Measurements were carried out after equilibrating the samples at a constant temperature. Shear viscosity measurements were performed by applying a ramp up and down of shear rate. Shearing time was set at t=20s in order to reach steady state. Before each test, a high rate steady shear (50 s⁻¹) was imposed to the suspension during 30s followed by a waiting time of 10min for the suspension to equilibrate at the set temperature. Long-time creep tests consisted of applying a constant stress (σ_c) during 600 s and recording the strain (γ), or equivalently the compliance J(t) = γ/σ_c .

The core volume fraction was calculated using the weight concentration c and the polymer density ρ (Eq.1). The polymer density was set at 1.2 g.cm⁻³ considering the density of a linear PEG polymer.

$$\phi_{\rm core} = \frac{c}{\rho} \tag{1}$$

The effective volume fraction was converted from the weight concentration by using a proportional factor determined with the dilute viscosity measurements. Curves and proportional factors for each system are presented in SI.

The microstructure of the microgels synthesized was analyzed by high-resolution transverse relaxation (T_2) NMR measurements using the protocol presented by other authors with some modifications.^{29,31} For these measurements a 2 wt% of microgels dissolved in deuterated water were used. All NMR experiments were performed at 25 °C on a Bruker Avance 400 spectrometer equipped with a Bruker 5 mm BBFO. The proton chemical shifts were referenced to the HOD signal at 4.70 ppm. Transverse relaxation (T2) values were determined using the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence using 10 values of spin–echo time (τ), with a minimum of 2 ms and a maximum of 1500 ms. The delay between 180° pulses in the CPMG block was 1.5 ms, and the number of scans was set at 64 and D1 at 5 s. The temperature was controlled by a Bruker temperature controller, which kept the sample temperature stable at 25°C.

Results & discussions

Swelling ability of microgels. The hydrodynamic diameters of microgels were analyzed by Dynamic Light Scattering (DLS) in order to evaluate the impact of the crosslinking density and distribution on the swelling ability. Figure 1 (A) and (B) shows the hydrodynamic diameters of OEGDA- and MBA-crosslinked microgels in a dilute solution at pH 6 for temperature ranging from 20°C to 60°C. As can be observed, microgels synthesized present the conventional thermal behavior, being highly swollen below VPTT and shrunken above the critical temperature due to the increase in the hydrophobic interactions between non-polar groups.^{22,23} The swelling ratios $\Phi_{20/60^{\circ}C}$, *i.e.* ratio of hydrodynamic diameter at 20°C over hydrodynamic diameter at 60°C, are summarized in Table S1. The less crosslinked microgels exhibit the highest swelling ratio, such as $\Phi_{20/60^\circ C} = 17.3$ for 2 mol.% OEGDA-crosslinked microgels whereas $\Phi_{20/60^\circ C} = 6.3$ for 8 mol.% OEGDA-crosslinked ones. The Volume Phase Transition temperature (VPTT) is not significantly influenced by the crosslinking density, as it was previously described in several studies on microgels and macroscopic gels.³⁸ However, an increasing crosslinking density induces a much more gradual volume phase transition as also reported in other studies. ³⁸⁻⁴⁰ A more gradual transition was ascribed by Woodward et al. to a decreased in the enthalpic energy of conformational change for higher crosslinker amount. The crosslinking points delay the conformational change of the thermoresponsive chains resulting in a larger range of temperatures corresponding to the VPPT. A more progressive VPT has also been ascribed to a more heterogenous structure, as previously reported by Pikabea et al. and Wu and Zhou.^{31,41} The increase in crosslinking content from 2 to 8 mol.% may enhance the heterogeneity of the microgel structure leading to a very progressive transition. OEGDA-crosslinked microgels exhibit a higher swelling ratio compared to MBA-crosslinked ones, regardless of the crosslinker content. With 8 mol. % of crosslinker, OEGDA swelling ratio, $\Phi_{20/60^{\circ}C}$, is around 6.3 compared to 3.1 for MBA one. As it was described by Imaz *et al.*⁴², OEGDA crosslinker (M_n =250 g·mol⁽⁻¹⁾) is a much longer spacer than MBA, which offers a higher flexibility to the microgel network and enhances its swelling ability. Furthermore, the microgels are more hydrophilic with the addition of OEGDA than MBA which lead to higher swelling abilities. The volume phase transition of 2-MBA is slightly more gradual than 2-OEGDA. As previously discussed, the gradual volume phase transition is commonly attributed to the heterogeneity in crosslinker distribution into the microgel network. Thus, the slightly broader phase transition of 2-MBA sample could come from a more heterogeneous distribution of the crosslinker molecules within the microgel.



Figure 1. (A) Hydrodynamic diameters *versus* temperature of 2-OEGDA and 2-MBA microgels in buffer of pH 6. (B) Hydrodynamic diameters *versus* temperature of 8-OEGDA and 8-MBA microgels in buffer of pH 6

Rheological behavior of microgels. Figure 2 shows the viscosity as a function of shear rate of 2 mol.% MBA-crosslinked microgels for weight concentrations between 5 wt. % and 20 wt.%. The data for the other systems can be found in supporting information (Figures S1-S3). At low

concentration, the suspensions behave as Newtonian fluids *i.e.* viscosity does not vary with shear rate. At higher concentration, the suspensions exhibit the typical shear rate dependence of concentrated colloidal suspensions, namely shear thinning and a plateau at high and low shear rates. No difference in viscosity is observed between the ramp up and the ramp down, which is characteristic of repulsive systems. Indeed, the negative charges coming from methacrylic acid units and the swollen dangling chains of microgels give rise to electro-steric repulsive forces that prevent particle attraction. Since particles do not aggregate, the shear rate-driven microstructure remains the same during both ramps. Figure 3 shows the rapid drop in viscosity for temperature decreasing from 14°C to 36°C for a 16.9 wt% 2-OEGDA sample. Indeed, a temperature approaching $\approx 32°$ C induces the collapse of microgels and the decrease of the occupied volume for a same particle density. Microgels are more distant from each other leading to the decrease of viscosity. Once the temperature is above the VPTT, viscosity almost ceases to decrease since the hydrodynamic diameter of microgels reaches a plateau. Viscosity curves at low concentration were fitted with an equation developed by Cross⁴³ in order to extrapolate the zero-shear and high-shear viscosities, η_0 and η_{er} respectively:

$$\eta = \eta_{\infty} + \frac{\eta_0 - \eta_{\infty}}{1 + (C\dot{\gamma})^m} \tag{2}$$

The fits according to Eq. (2) are represented by the dashed lines in Figure 2. The zero-shear viscosity is used to calculate the relative viscosity η_r expressed by $\eta_r = \eta_0 / \eta_s$, with η_s the solvent viscosity (taken at 0.001 Pa.s for Milli-Q water). To compare the systems, the relative viscosity is plotted as a function of the volume fraction in Figure 4 for the four suspensions. Since the swelling is not considered in the core volume fraction parameter, the viscosity diverges at a critical volume fraction ϕ^* much lower than the hard-sphere one, commonly taken at ≈ 0.58 for

repulsive glass.⁴⁴⁻⁴⁶ The viscosity diverges at a higher volume fraction for increasing crosslinking densities of microgels. The influence of the crosslinker and the crosslinking density have been described in several studies.^{16,47–49} In theta solvent, microgels are generally described as highly swollen and deformable spheres with a soft and fuzzy corona layer. As the concentration increases, those swollen microgels approach from one another resulting in a rapid increment of viscosity. It was repeatedly observed that for a same weight concentration, the viscosity of highly crosslinked microgels is much lower than the one of lightly crosslinked microgels. It was suggested that the lightly crosslinked microgels present longer-range interaction at larger separation distance due to their looser structure and softer steric layer thus, resulting in a higher viscosity. The increase in the degree of crosslinking reduces the swelling and hinders the microgel inter-particle bridging interaction.⁴⁸ Therefore, microgels crosslinked with the higher crosslinker rate (8 mol.%) resemble more to hard spheres and the viscosity diverges closer from the theoretical value of 0.58, such as observed in other work.¹⁶ The type of crosslinker also strongly affects the rheological behavior of the suspensions. The zero-shear viscosity of 2-MBA sample diverges at a lower concentration than the 2-OEGDA one, and equivalently for 8 mol.% of crosslinker. This result is not in agreement with the DLS measurements that reveal a higher swelling ratio for OEGDA for both crosslinker rates (see Figure 1). At a theoretically equal crosslinker rate, one would expect MBA samples to diverge at higher volume fraction considering their lower swelling ability. This discrepancy could come from a different topology of the outer microgel shell induced by the different distribution of the crosslinking points within the particle being the shell of the microgels synthesized with MBA less crosslinked than that of OEGDA ones. This part will be further discussed by screening the microgel microstructure by NMR.



Figure 2: Viscosity as a function of shear rate for different weight concentrations of a 2-MBA sample. Ramp up and ramp down. The dashed lines represent the fits according to Cross Equation, Eq. (2).



Figure 3: Viscosity as a function of shear rate for different temperatures of a 16.9 wt% 2-OEGDA sample. Ramp up and ramp down.

Microgels are compared to the rheological behavior of hard-sphere models by scaling the relative viscosity against the effective volume fraction ϕ_{eff} in Figure 4 (B). The effective volume fraction was determined through the measurements of the relative viscosity in dilute concentrations as commonly described.⁴⁷ The curves of the relative viscosity in dilute concentration are shown in Figure S6 for the four systems. One can outline that the limit of this method, based on measurements in dilute concentrations, is not to consider the eventual deswelling of microgels at high packing fraction. Indeed, microgels can undergo deswelling as the particle concentration increases due to steric compression of the overlapping chains between particles.⁵⁰ Thus, the effective volume fraction is surely less accurate at high packing fraction. The inherent difficulty to assess the value of the volume fraction regardless of the chosen method highlights the complexity of soft microgel systems compared to hard sphere, as reported by Lyon et al.¹⁵

As observed in Figure 4 (B), the relative viscosity curves of the four systems (2-MBA, 2-OEGDA, 8-MBA and 8-OEGDA) successfully reduce into a single master curve when plotted against ϕ_{eff} . The relative viscosity of concentrated hard sphere suspensions is commonly described by the Eq (3) named the Quemada equation:⁵¹

$$\eta_r = (1 - \frac{\phi}{\phi_{max}})^{-2} \tag{3}$$

Where ϕ_{max} is usually set at 0.64 for crystals and 0.58 for glass suspensions.

Or Eq. (4) named the Krieger-Dougherty equation:⁵²

$$\eta_r = (1 - \frac{\phi}{\phi_{max}})^{-[\eta]\phi_{max}} \tag{4}$$

The Quemada equation with $\phi_{max} = 0.58$ and $\phi_{max} = 0.64$ is represented by the two black lines in Figure 4 (B). The different microgel suspensions are fairly well described by the hard sphere model until ≈ 0.45 , regardless of the crosslinker type and density. It indicates that microgels can be seen as impermeable hydrodynamic particles in their non-draining behavior. At these crosslinking densities, the solvent is not flowing through microgels but moving along with its polymer chains. Data points from the most crosslinked suspensions, 8-OEGDA and 8-MBA, run particularly close to the Quemada hard sphere model. As one expects, the more the microgels are crosslinked, the more they resemble to hard spheres. The softer suspensions, 2-MBA and 2-OEGDA, diverge at a slightly lower effective volume fraction than the colloidal glass transition of hard spheres at 0.58. The samples are better described by a Krieger-Dougherty equation, represented by the dashed line in Figure 4 (B), for which ϕ_{max} is set at 0.48 and [η] at 3. The early divergence of the relative viscosity of these samples is not in agreement with the divergence at high φ_{eff} usually described by the literature for soft systems.¹²⁻¹⁴ Although the reason of the divergence at higher ϕ_{eff} is commonly attributed to the deformation of soft particles, some authors stated it could also be caused by high polydispersity.⁴⁷ The polydispersity indexes measured with DLS at 20°C, are found to be lower than 5% for the four samples, as seen in Table S2. Such low polydispersity is suitable for a fluid-crystal coexistence regime, since previous studies have demonstrated that polydispersity should be higher than $\approx 8\%$ to prevents the formation of crystal regions.^{9,10,53,54} Yet, the polydispersity indexes being very similar for the four systems, the early viscosity divergence seems to be driven by the microstructure of the softer microgels. One can suppose the numerous dangling chains of the microgel shell may hinder the particle motion by creating friction and interpenetration as soon as particles are packed. The particle deformation occurs at higher concentrations, when the system is already dynamically arrested by friction and interpenetration.



Figure 4: (A) Relative viscosity as a function of the core volume fraction for 2-MBA, 2-OEGDA, 8-MBA and 8-OEGDA samples (B) Relative viscosity as a function of effective volume fraction for 2-MBA, 2-OEGDA, 8-MBA and 8-OEGDA samples. The solid and dashed lines represent the hard sphere behavior according to Quemada equation with respectively ϕ_{max} = 0.58 and ϕ_{max} = 0.64. The dotted line corresponds to the Krieger-Dougherty equation where the parameters were set to obtain the best fit, accordingly ϕ_{max} = 0.48 and [η]=3.

Stress data from shear viscosity measurements are plotted against shear rate in Figure 5 (A) and (B) for respectively 2-MBA and 2-OEGDA sample. The data for 8-OEGDA and 8-MBA can be found in supporting information (S4 and S5). Above the critical volume fraction at which viscosity diverges, the suspensions exhibit elastic properties including a yield stress. The curves

above ϕ^* are fitted by a Herschel-Bulckley model which is known to well described the rheological behavior of simple yield stress fluids as proposed in Eq. 5:

$$\sigma = \sigma_{\rm v} + K_{\rm HB}(\dot{\gamma})^{\rm n} \tag{5}$$

with σ_y the yield stress, K_{HB} the consistency index and *n* the flow index. The difference induced by the crosslinker type is even more pronounced for volume fraction above ϕ^* , once the particles are densely packed. As a comparison, for a similar concentration of ≈ 18 wt. %, the yield stress, extrapolated from the HB fit, of 2-MBA is about 50 times greater than 2-OEGDA one, respectively 5.72 Pa and 0.12 Pa.

However, rather than measuring shear viscosity, elastic properties of repulsive glasses are most commonly characterized with oscillatory shear or creep measurements which represent the proper method to determine a yield stress.^{7,55–59} In previous works, Senff and Richtering⁴⁷ A indicated that the yield stress was more relevant to reveal the chain topology at the microgel shell whereas the moduli in oscillatory shear are more dependent on the crosslinking degree within the microgels. Thus, long-time creep tests were performed in order to further investigate the hypothesis of a looser crosslinked shell in MBA samples.



Figure 5: Stress as a function of shear rate for different weight concentrations of (A) 2-MBA suspension (B) 2-OEGDA suspension. The solid lines are fits according the Herschel-Bulckley equation.

The compliance vs time curves are represented in Figure 6 (A) and (B) for 2-OEGDA and 2-MBA samples at a concentration of 18 wt. %. Increasing transient stresses were applied until the suspensions exhibit complete flow. The oscillations at the start of a creep test, especially observed for 2-OEGDA sample, come from the instrument inertia coupled with the sample elasticity.⁶⁰ For stresses greater than the yield stress σ_y , the increase of strain is linear with time. The slope of 1 in the log-log plot expresses the fully developed flow of the suspensions. For stresses smaller than $\sigma_{\rm y}$, the strain does not reach a plateau with long time, as it could be expected for an ideal solid. It increases in a sublinear manner without reaching a fully developed flow. For the sake of comparison, the last decades of the compliance curves are fitted by an empirical power-law: J(t) \propto t^{λ}, as described by Pham *et al.*⁵⁹, although no theoretical justification supports its use. The increase of the exponent value illustrates well the solid-to-liquid transition. The complete flow of the system is described by $\lambda = 1$, and the associated stress can be taken as the yield stress. As observed in the semi-log plot in Figure 6 (C), the yield stress of 2-MBA corresponds to 5.00 Pa, whereas the yield of 2-OEGDA occurs at 0.05 Pa. Both values corroborate well with the yield stresses extrapolated from shear viscosity data, respectively 5.72 Pa and 0.12 Pa. A much greater stress is required to break down the particle assembly of the 2-MBA sample, revealing a stronger network of microgels. As it is well known, at such a highly concentrated state, microgels deform and interpenetrate between each other. These results strengthen the idea that the shell of 2-MBA has a higher capacity to interpenetrate. Most likely, the shell is composed of longer and loosely crosslinked dangling chains that are more suitable for entanglement. Yet, the samples undergo a similar yielding mechanism, although not occurring at the same value. Indeed, the evolution of λ with σ_c is sharp from 0 to 1, describing an abrupt transition from creep to flow for both 2-MBA and 2-OEGDA. This is characteristic of repulsivedominated suspensions, where the sharp transition is physically interpreted by the sudden cage breaking.^{61–63} Despite the friction due to chain entanglements, the electro-steric repulsive forces, generated by the negative charges and the swollen dangling chains, dominate the yielding mechanism. Further structural characterization was undertaken by ¹H NMR transverse relaxation measurements to validate our hypothesis and quantitatively investigate the microstructure induced by the crosslinkers.







Figure 6: Compliance curves as a function of time for different constant stresses applied for (A) 2-MBA suspension (B) 2-OEGDA suspension. (C) Exponent λ from the power law J(t) $\propto t^{\lambda}$ versus the applied stresses for 2-MBA and 2-OEGDA samples

Microstructure of microgels. With the aim of linking the particle microstructure to macroscopic properties, ¹H-nuclear magnetic resonance (NMR) transverse relaxation measurements were carried out. To investigate the heterogeneity in microstructure of the different microgel particles, the resonance peak that appears at 3.6 ppm of methylene protons of (PMeO₂MA-OEGMA) chains was used due to its high intensity (see Figure S1). Biexponential decay for the integral of methylene protons for the different microgels was assumed. Then, using the equations suggested by Balaceanu *et al.*,²⁹ short (T_{2S}) and long (T_{2L}) transverse relaxation times together with the relative amounts of methylene protons of (PMeO₂MA-OEGMA) chains in core (C_S) and shell (C_L) were obtained describing quantitatively the bimodal heterogeneity of the polymer network in microgel particles. In addition, the ratio of the crosslinking densities (CLD^{core}/CLD^{shell}) of the microgels was obtained from eq (6):

$$\frac{CLD^{core}}{CLD^{shell}} = \left(\frac{T_{2L}}{T_{2S}}\right)^{1/2} \tag{6}$$

The relative amounts of methylene protons of (PMeO₂MA-OEGMA) (C_S and C_L) and the ratios of crosslinking densities (CLD^{core}/CLD^{shell}) are reported in Table 1. As can be observed, regardless of the type and amount of crosslinker used, the C_S and C_L values are similar, in all the cases. This expected result can be understood considering the polymerization kinetics of MeO₂MA and OEGMA monomers. Boularas *et al.*²² reported that the consumption rates of both monomers were not affected by the crosslinker type leading to a similar distribution of PMeO₂MA-OEGMA chains into the microgel particles. Similar results were reported by other authors in the case of poly(vinylcaprolactam) (PVCL)²¹ and poly(2-diethylaminoethyl) methacrylate (PDEAEMA)²⁷ -based nanogels.

Table 1. Relative weight coefficient of (PMeO₂MA-OEGMA) chains in the core (C_S) and shell (C_L) and crosslinking density ratios (CLD^{core}/CLD^{shell}) of microgels cross-linked with OEGDA and MBA.

Crosslinker	Cs CL		CLD ^{core} /CLD ^{shel}				
OEGDA (2 mol %)	0.49	0.5 7	5.3				
MBA (2 mol %)	0.5 1	0.5 7	3.5				
OEGDA (8 mol %)	0.4 6	0.6 0	3.8				
MBA (8 mol %)	0.5 1	0.5 1	3.4				

Regarding crosslinking density ratios, a core-shell microstructure is obtained in all the cases. Moreover, an effect of the crosslinker type and amount on the crosslinking density ratio and thus on the heterogeneity of the morphology is observed. In this sense, the lowest crosslinking density ratio was obtained by using MBA as crosslinker. The reason of that could be the difference between the reactivities of both crosslinkers (MBA and OEGMA) when they copolymerize with methacrylates, in this case, MeO₂MA and OEGMA. To confirm this hypothesis, the reactivity ratios of MBA and OEGDA with respect of OEGMA were determined by ¹H NMR following the method described by Mahdavian *et al.*⁶⁴ It was determined that MBA (r_{OEGMA} = 1.84 and r_{MBA} = 0.98) is less reactive than OEGMA (r_{OEGMA} = 1.19 and r_{OEGDA} = 1.12) when copolymerized with OEGMA leading to a more homogeneous core-shell structure. In addition, increasing the amount of MBA no effect on crosslinking density is observed. This means that the

ratio between core and shell, in terms of mobility, is not change with the increase in the amount of MBA. The same behavior was obtained by Balaceanu *et al.*²⁹ for PVCL-based microgels prepared with different amounts of MBA as a crosslinker. In the case of OEGDA crosslinker, increasing its concentration, the crosslinking ratio decreases from 5.3 to 3.8 being more similar to the values obtained in the case of using MBA as crosslinker.

Moreover, the study of the microgels microstructure could be related with the results obtained analyzing the rheological properties of microgel suspensions. As mentioned before, microgels particles cross-linked with MBA present better interpenetration with a strong capacity to assemble into structured network than OEGDA cross-linked microgel particles in all the crosslinker concentration range studied. This could be due to the crosslinking density difference observed in both types of microgels. In the interpenetration process, the shell of the particles is more involved than the core. In the case of microgels cross-linked with MBA the crosslinking density of the shell could be lower than that of OEGDA cross-linked ones leading to an easier interpenetration of the microgel particles. With the aim of corroborating this hypothesis, the Flory-Rehner theory was applied to the microgel synthesized.

In this regard, Balaceanu *et al.* reported an extended Flory-Rehner theory for thermoresponsive microgels based on a bimodal crosslinking density model.^{29,30} Recently, Pikabea *et al.* applied the extended Flory-Rehner theory to analyze the inner structure of thermo- and pH-responsive nanogels.³¹ Those authors reported that number of chains between two crosslinking points, *i.e.* subchains, was proportional to the relative weight coefficients of polymeric chains (C_L and C_S) and the relaxation times (T_{2S} and T_{2L}) measured by T_2 NMR experiments as described by Eq. (7):

$$\frac{N^{core}}{N^{shell}} = \frac{C_S}{C_L} \left(\frac{T_{2L}}{T_{2S}}\right)^{1/2} \tag{7}$$

Moreover, the number of subchains in the microgel particles can be estimated considering that each crosslinker molecule connects two subchains with the Eq. (8):

$$N = 2N_A v_0 c \tag{8}$$

where N_A is the Avogadro's constant, v_0 is the volume of each microgel particle and c is the molar concentration of crosslinker. Using the data obtained from above formulas, it is possible to calculate the proportionality parameter (ρ) between the radius of the core and the hydrodynamic radius of the microgel particles from the Eq.(9):

$$N = \frac{1}{2} \left(\frac{N^{core}}{\rho^3} + \frac{N^{shell}}{1 - \rho^3} \right)$$
(9)

Table 2 shows the number of subchains in the core (N^{core}), number of subchains in the shell (N^{shell}) and the ratio between the radius of the core and the hydrodynamic radius of the microgel particles (ρ). It is important to point out that the parameter ρ is assumed to be temperature and pH independent.^{29,31} As can be observed, in the case of microgels synthesized using MBA as crosslinker, the number of subchains in the shell is lower being this difference more important in the case of microgel synthesized with 8 mol% of crosslinker. Thus, although for both microgels the shell of the particles is less crosslinked allowing the interpenetration of the particles, in the case of the ones cross-linked with MBA the crosslinking density of the shell is even lower than that of OEGDA ones leading to a stronger capacity to assemble among them corroborating above hypothesis. These data do not agree with the hypothesis reported by Boularas *et al.*²¹ suggesting a highly crosslinked shell and slightly crosslinked core in the case of microgels crosslinked with

MBA. They observed an impact of the crosslinker type on kinetic data by NMR during the analysis of non-purified microgel dispersions maybe because the impurities present in the dispersion could influence the results obtained. By contrast, in this work, purified microgel dispersions have been used during the determination of microgels microstructure and therefore, any external effect has been avoided. In summary, these results confirm that the different distribution of crosslinking points affects the rheological properties of microgel suspensions such as MBA samples exhibit a higher yield stress than OEGDA samples.

Table 2. Number of subchains in the core (N^{core}) and in the shell (N^{shell}) and the parameter ρ as a function of crosslinker type and amount (derived from Eq. (9)).

Crosslinker	N ^{core}	N ^{shell}	ρ	
OEGDA (2 mol %)	13760	2870	0.87	
MBA (2 mol %)	8480	2750	0.85	
OEGDA (8 mol %)	1591200	600440	0.86	
MBA (8 mol %)	456640	134300	0.86	

In addition, similar values of the parameter ρ are obtained despite of the cross-linked type and amount used. The value of $\rho \approx 0.86$ means that the relative volume of the core is 66% of the total volume of the microgel particles. This value is much lower than that observed by Pikabea *et al.* for PDEAEMA-based nanogel particles (82%).³¹ This larger shell could be one of the reasons of

the trigger-free spontaneous film formation properties presented by the microgels synthesized. These films present colored properties¹ and unusually high encapsulation values for different active molecules² being suitable for cosmetic and healthcare or medicine applications.

Conclusions

On the basis of the previously reported techniques for the microgels microstructure study ²³⁻²⁷, this work has demonstrated that differences in crosslinking density can be studied by coupling rheological tests and ¹H NMR transverse relaxation measurements.

The zero-shear viscosity and long-time creep tests on concentrated microgel suspensions have proved to be suitable techniques to reveal the differences in microstructure. Indeed, at higher crosslinking density, microgels resemble more to hard spheres and consequently, the relative viscosity diverges at a higher core volume fraction¹⁵. For both crosslinking rates, the viscosity of OEGDA samples diverges at a higher concentration than MBA ones, suggesting a looser crosslinked shell for MBA-crosslinked microgels. Creep tests revealed a higher yield stress for MBA samples than OEGDA ones for a same concentration, respectively 5 Pa and 0.05 Pa. These results strengthen the hypothesis of a looser crosslinked shell for MBA microstructure, which enables more interpenetration between particles, and thus leads to a stronger network. Yet, the transition from solid-to-liquid was sharp for both crosslinkers, indicating the microstructure did not affect the yielding mechanism⁵⁷⁻⁵⁹. In view of the small yield stress values, it can be noted that the suspensions, at this concentration, have very weak solid properties since an order of few Pascals is enough to trigger the solid-to-liquid transition. In this work, it was also demonstrated that the effective volume fraction is an appropriate scaling factor to compare microgels to the

hard sphere behavior. Indeed, the viscosity curves successfully reduce into a single curve and closely follow hard sphere models such as the Quemada relationship⁴⁷ up to $\phi_{eff} < 0.45$. The viscosities of softer suspensions do not diverge at higher ϕ_{eff} than the colloidal glass transition value, which is usually the case for soft systems¹²⁻¹⁴. One can suppose the numerous dangling chains of the microgel shell may hinder the particle motion by creating friction and interpenetration as soon as particles are densely packed. In this case, the particle deformation occurs when the system is already frozen, at higher concentrations. Finally, transverse relaxation (T₂) NMR measurements demonstrated that microgels exhibit a core-shell microstructure for both crosslinker types and both crosslinking densities, similar to what was observed for PVCL microgels crosslinked with different amounts of MBA²⁸. Yet, MBA samples present a lower number of subchains in the shell, attesting of a less crosslinked shell. This result validates the hypotheses made in view of the rheological results.

In summary, the effect of different crosslinking architectures on particles assembly in suspension has been determined being the next step the study of its effect on self-assembled microgels film mechanical properties. Indeed, we already demonstrated during the solvent evaporation that the suspensions evolve from a fluid to a cohesive film thanks to the self-assembly of microgels³¹ without characterization of the viscoelastic properties of the films towards the skin properties.

Associated comments

Swelling ratios and polydispersity indexes of microgels synthesized, viscosities and stresses of different microgels, relative viscosities of different microgels, proton spectrum of microgels cross-linked with OEGDA.

Author information

Corresponding Author

*E-mail: laurent.billon@univ-pau.fr; Tel: +33 559407609

*E-mail: christophe.derail@univ-pau.fr; Tel: +33 559407706

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

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1	Simulation and experimental study of enzyme and reactant mixing in capillary					
2	electrophoresis based on-line methods					
3						
4	Marta Pelcová ^{a,b} , Roman Řemínek ^{a,b} , Friederike A. Sandbaumhüter ^a , Richard A. Mosher ^c ,					
5	Zdeněk Glatz ^b , Wolfgang Thormann ^{a,*}					
6						
7	^a Clinical Pharmacology Laboratory, Institute for Infectious Diseases, University of Bern, Bern,					
8	Switzerland					
9	^b Department of Biochemistry, Faculty of Science, Masaryk University, Brno, Czech Republic					
10	^c RAM Software Solutions, Tucson, AZ, USA.					
11						
12						
13						
14						
15						
16						
17						
18						
19						
20 21	*) Correspondence:					
22	Prof. Dr. Wolfgang Thormann					
23	Clinical Pharmacology Laboratory, Institute for Infectious Diseases, University of Bern,					
24	Murtenstrasse 35, 3008 Bern, Switzerland					
25	E-mail: wolfgang.thormann@ifik.unibe.ch, Tel: +41-31-632-32-88, Fax: +41-31-632-4997					

27 Abstract

28	The establishment of an efficient reaction mixture represents a crucial part of capillary
29	electrophoresis based on-line enzymatic assays. For ketamine N-demethylation to norketamine
30	mediated by the cytochrome P450 3A4 enzyme, mixing of enzyme and reactants in the
31	incubation buffer at physiological pH was studied by computer simulation. A dynamic
32	electrophoretic simulator that encompasses Taylor-Aris diffusivity which accounts for dispersion
33	due to the parabolic flow profile associated with pressure driven flow was utilized. The simulator
34	in the diffusion mode was used to predict transverse diffusional reactant mixing occurring during
35	hydrodynamic plug injection of configurations featuring four and seven plugs. The same
36	simulator in the electrophoretic mode was applied to study electrophoretic reactant mixing
37	caused by voltage application in absence of buffer flow. Resulting conclusions were
38	experimentally verified with enantioselective analysis of norketamine in a background
39	electrolyte at low pH. Furthermore, simulations visualize buffer changes that occur upon power
40	application between incubation buffer and background electrolyte and have an influence on the
41	reaction mixture.
42	
43	
44	
45	
46	
47	Key words: capillary electrophoresis, enzyme reaction, electrophoretically mediated
40	incroanarysis, unrusion, sinuration, rayior-Aris dispersion

49

50 **1. Introduction**

51 During the last three decades, capillary electrophoresis (CE) became a powerful technique 52 providing accurate and highly efficient analyses in many areas, including drug metabolism 53 studies, requiring only minuscule amounts of sample and chemicals. The commonly used CE-54 based enzyme assay modes comprise two categories, namely (i) pre-capillary (off-line) assays in 55 which the reaction mixture is incubated in a vial and the reaction products are analyzed by CE, 56 and (ii) in-capillary (on-line) assays where incubation of a reaction mixture and analysis of 57 reaction products are carried out in the capillary. Due to the small inner diameter of the capillary, 58 the latter approach provides true nanoliter-scale assays and fulfills the demand of the 59 pharmaceutical industry for miniaturized, fully automated screening systems [1,2]. An on-line 60 method typically consists of four steps, consecutive injection of enzyme and reactant solution 61 plugs and their mixing, incubation of reaction mixture, application of voltage and detection of 62 reaction products. The lengths of the injected plugs are one to two orders of magnitude larger 63 compared to the inner diameter of the capillary. In combination with liquid incompressibility, 64 this makes perfect mixing of individually injected plugs difficult and thus represents a bottleneck of on-line CE methods for activity, kinetic and inhibition studies of enzymes. Incomplete mixing 65 provides heterogeneous distribution of enzyme and reactants and thus regions of different 66 67 effective reaction rates leading to inaccurate values of pharmacokinetic and enzymatic 68 parameters. Assessment of reaction mixture distribution is thus a crucial part of on-line CE 69 assay.

70 In practice, plug injection effected by application of differential pressure constitutes an inherent

71 part of the mixing process and determines the initial distribution of enzyme and reactants inside

the capillary. Several processes contribute to mass transfer during injection, including

73 translational movement along the capillary, diffusion along the capillary, and diffusion across the

capillary. Due to the friction close to the capillary inner wall, each injected plug possesses a

characteristic parabolic profile which can be described by the Poiseuille equation (Fig. 1). With

- respect to mixing principles, assays can be divided into two groups. Firstly, mixing of enzyme
- and reactants can be obtained with electrophoretic merging, a process referred to as
- relectrophoretically mediated microanalysis (EMMA) [3,4]. The other method is based on
- ransverse diffusion of laminar flow profiles (TDLFP) proposed by the group of Krylov [5,6].
- 80 This approach relies on the application of a relatively high differential pressure for a short time

81 period which makes the plugs strongly parabolic. Each injected plug penetrates into the

- 82 preceding zone thereby creating longitudinal interfaces (Fig. 1). Even large molecules, such as
- 83 enzymes, are able to diffuse across the distance of capillary radius or diameter in tens of seconds

84 such that a relatively homogeneous reaction mixture is quickly formed by transverse diffusion

85 [5-9]. Mixing by transverse diffusion can be studied with the TDLFP model of Krylov in Excel

86 [5-8]. Another approach, reported here for the first time, is the use of a dynamic electrophoretic

- 87 simulator in the diffusion mode. Electrophoretic simulators provide component distributions
- 88 within the capillary as function of time [10-16]. Two one-dimensional simulators, GENTRANS
- 89 [12] and SPRESSO [14], feature a module that calculates zone and boundary dispersion caused
- 90 by hydrodynamic flow and molecular diffusion using the Taylor-Aris equation [17,18]

91
$$D_{i(eff)} = D_i \left[1 + \beta P_e^2\right]$$
 with $P_e = Ud/D_i$ (1)

92 where $D_{i(eff)}$ is the effective diffusion coefficient, D_i is the diffusion coefficient of species *i* in 93 absence of pressure driven flow (molecular diffusion), *U* is the average velocity of the pressure 94 driven flow, *d* is the inner diameter of the capillary, and β is a constant representing the cross 95 sectional shape of the capillary. For a circular capillary with diameter *d*, $\beta = 1/192$ [19,20].

In a previous study, Stahl et al. used a dynamic electrophoretic simulator to study plug-plug
mixing phenomena in EMMA associated with local conductivity changes [21]. This work
demonstrates the importance of employing a simulator which considers changes in the electric
field in order to understand sample mixing in EMMA. Dynamic EMMA computer models which
provide insight into electrophoretic mixing of reactants in a uniform electric field do not consider

- 101 changes of buffer composition induced by the electric field [22-24]. The goal of the presented
- 102 study was to characterize the mixing of the enzyme and the reactants in the two on-line

103 configurations presented in Fig. 1 using dynamic computer simulation. These arrangements were

- 104 previously employed for studying the enantioselective metabolism of ketamine mediated by the
- 105 cytochrome P450 3A4 isoform (CYP3A4) enzyme. The four-plug configuration of Fig. 1A was
- 106 used with voltage applied for a short time prior to incubation [25] and the seven-plug
- arrangement of Fig. 1B was employed in the TDLFP format [26]. Incubation occurred at
- 108 physiological pH and analysis of formed norketamine enantiomers was achieved in a low pH
- 109 background electrolyte (BGE) in presence of a negatively charged chiral selector. For both

110 systems, diffusional reactant mixing occurring during hydrodynamic plug insertion was analyzed 111 with the GENTRANS simulator in the diffusion mode and compared to that predicted with the 112 TDLFP model of Krylov. For the four-plug configuration, GENTRANS in the electrophoresis 113 mode was employed to study the effect of power application on mixing after plug insertion, the 114 use of alternative initial plug arrangements, and the impact of buffer changes occurring upon 115 current flow at the discontinuity between incubation buffer (IB) and BGE. Experimental data 116 were assessed on the basis of enantioselective norketamine analysis in the two on-line formats 117 and compared to the predictions obtained by simulation. Furthermore, application of the cofactor 118 reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as pure compound or with a 119 regenerating system was also examined.

120 **2. Materials and methods**

121 2.1. Chemicals, reagents and solutions

122 Racemic ketamine and norketamine (as hydrochloride solutions in methanol, 1 mg/mL of the 123 free base) were from Cerilliant (Round Rock, TX, USA). NADPH was purchased from Sigma-124 Aldrich (Buchs, Switzerland) as tetra sodium salt. Phosphoric acid (85%), potassium dihydrogen 125 phosphate, and di-potassium phosphate were from Fluka (Buchs, Switzerland), and Tris from 126 Merck (Darmstadt, Germany). Highly sulfated γ-cyclodextrin (HS-γ-CD) (20%) was purchased from Beckman Coulter (Fullerton, CA, USA). SUPERSOMESTM containing human CYP3A4, 127 128 P450 reductase and cytochrome b5 and the NADPH regenerating system (NADPH-RS) were 129 obtained from BD Gentest (Woburn, MA, USA; distributed through Corning, Wiesbaden, Germany). The NADPH-RS comprises two solutions, solution A with NADP+, glucose-6-130 131 phosphate and MgCl₂ and solution B with glucose-6-phophate dehydrogenase in sodium citrate. 132 The enzyme solution was divided into aliquots and stored at -80 °C whereas NADPH-RS 133 aliquots were kept at -20 °C. A stock solution of 10 mM NADPH was titrated to pH 8.0 with 134 addition of 0.1 M NaOH and stored as aliquots at -20 °C. The pH 7.4 IB was prepared with 100 135 mM solutions of K_2 HPO₄ and KH₂PO₄. The plug solutions were prepared daily in IB. The plug 136 containing NADPH-RS was composed of solution A (25.00 %), solution B (5.00 %), IB (61.00

- 137 %), ketamine solution (7.15 %) and 1 M NaOH (1.85 %) [25].
- 138 2.2. Capillary electrophoresis

140 photodiode array UV-VIS detector at 195 nm was used for all experiments and analyses were carried out as described before [25,26]. Briefly, the bare 50 µm id (375 µm od) fused-silica 141 142 capillaries (Polymicro Technologies, Phoenix AZ, USA) were of 0.45 m total length (0.35 m 143 effective length, [25]) and 0.60 m of total (0.50 m effective length, [26]). BGE was composed of 144 50 mM Tris-phosphate buffer (pH 2.5) and HS- γ -CD as a chiral selector (2 % v/v [25] and 3 % 145 v/v [26]). The four-plug approach included a 3.5 min cooling period between the incubation 146 (37°C) and separation (25°C) [25], whereas the seven-plug method was executed at 37°C [26]. 147 Separations were performed by concomitant application of voltage (four-plug method: -12 kV, 148 266.7 V cm⁻¹, observed current about -41 µA; seven-plug method: -20 kV, 333.3 V cm⁻¹, about -149 $86 \,\mu\text{A}$) and positive pressure towards anode (0.1 psi (0.7 kPa) for both approaches). Data was 150 acquired and peak areas were determined using the 32 Karat 7.0 software (Beckman Coulter).

A ProteomeLab PA 800 CE System (Beckman Coulter, Fullerton, CA, USA) equipped with a

151 Results were reported as corrected peak areas, i.e. peak areas divided by migration time (units:

152 μAU).

139

153 2.3. Conditions for in-capillary reaction

154 For the four-plug configuration of Fig. 1A and if not stated otherwise, each plug was injected by 155 application of 1 psi (6.9 kPa) for 4 s, providing plug volumes of about 13.6 nL each. The overall 156 length of the entire four-plug assembly was calculated to be approximately 27.6 mm (7.9 % of 157 total capillary length). Furthermore, in selected experiments, application of -10 kV (222.2 V/cm) 158 for 12 s (ramping for 10 s) was applied after insertion of the plugs. The configuration of Fig. 1B 159 comprised an alternate injection of 4 plugs of ketamine and NADPH solution and 3 plugs of 160 CYP3A4 solution. Each plug of the substrate and cofactor was injected for 3 s into the capillary at a pressure of 0.5 psi (3.45 kPa) and each plug of CYP3A4 SUPERSOMES was introduced by 161 162 application of a negative pressure of -0.5 psi (-3.45 kPa) for 4 s. The use of negative pressure for 163 CYP3A4 SUPERSOMES insertion provided better repeatability of the injection procedure [26]. 164 The calculated plug volumes were approximately 3.8 and 5.1 nL, respectively. The estimated 165 volume of the entire seven-plug assembly thus was 30.6 nL (15.5 mm or 3.1 % of total 166 capillary). In both methods, the inlet capillary tip was washed by dipping in IB after each 167 injection in order to minimize the mass transfer between the vials of the enzyme and reactants.

168 Incubation time intervals at 37 $^{\circ}$ C used for the four- and seven-plug approaches were 8 and 10

- 169 min, respectively. The reference samples of norketamine production were prepared by off-line
- 170 incubation of CYP3A4, ketamine and NADPH at the same concentration as was estimated for
- 171 on-line assays. The reaction mixtures were incubated for a respective time period at 350 rpm and
- 172 37°C in a thermomixer (Eppendorf, Germany).
- 173 2.4. Tools for simulation, input data, data evaluation and execution of simulations
- 174 All computations were executed on Windows 7-based PCs with a 32bit operation system. The
- reactants distribution in the capillary was investigated by GENTRANS using the version
- 176 comprising Taylor-Aris diffusivity which accounts for dispersion due to the parabolic flow
- 177 profile [12] and the TDLFP model of Krylov in Excel [6] which was downloaded from
- 178 www.chem.yorku.ca/profs/krylov. Electrophoretic mobilities of reactants in IB were determined
- by CE using standard methods and short end injection according to Williams and Vigh [27].
- 180 Sample plug lengths and the velocity of the imposed applied flow were estimated with the CE
- 181 Expert Lite software (Beckman Coulter, Fullerton, CA, USA) and buffer compositions, ionic
- 182 strength and component mobilities were calculated using Peakmaster 5.2 software (downloaded
- 183 from http://www.natur.cuni.cz/Gas [28]). SigmaPlot 12.5 (Systat Software, San Jose, CA, USA)
- 184 was used for data presentation and statistical analysis of experimental data.
- 185 For the simulations with GENTRANS, NADPH was applied as cofactor and a protein with a pI
- 186 of 8.6 represented the CYP3A4 SUPERSOMES. If not stated otherwise, the plugs comprised IB
- 187 composed of 100 mM H₃PO₄ and 161 mM KOH with a calculated pH of 7.40 together with
- 188 either 400 nM of a protein (CYP3A4 SUPERSOMES) or 1 mM ketamine hydrochloride together
- 189 with 2 mM NADPH or no additional components. The BGE was composed of 77 mM H₃PO₄, 50
- 190 mM Tris, 7.6 mM of a negatively charged chiral selector and 7.6 mM sodium from the chiral
- selector. The calculated pH of the BGE was 2.51. Physico-chemical input constants used are
- summarized in Table 1. Initial zone structures with plugs of realistic lengths and sharp
- 193 boundaries were established and shifted along the 50 μ m ID capillary for specified time intervals
- 194 with and without applying Taylor-Aris diffusivity. The capillary length was 10 cm and divided
- 195 into 10000 segments ($\Delta x = 10 \ \mu m$).
- 196 **3. Results and discussion**

197 3.1. Simulation of diffusional mixing during plug injection

198 GENTRANS was employed to estimate diffusional mixing of the enzyme and the reactants for 199 the four-plug and seven-plug configurations of Fig. 1 using injection velocities of 1800 µm/s and 200 600 µm/s, respectively. Computer predicted concentration profiles for protein, ketamine and 201 NADPH inside the capillary introduced by the four-plug injection procedures of Fig. 1A and the 202 seven-plug method of Fig. 1B are shown in Fig. 2A and 2B, respectively. Profiles depicted are 203 those for 16 s and 24 s, respectively, the time intervals used for insertion of the plugs in the two 204 approaches. Distributions of the buffer components are not presented. Initial distributions of the 205 protein and the reactants are depicted as inserts on the left hand side of each panel and the 206 profiles predicted without Taylor-Aris diffusivity, i.e. with molecular or longitudinal diffusion 207 only, for the respective time intervals are shown as inserts on the right side in each panel. These 208 data indicate that mixing by transverse diffusion is effective whereas mixing by longitudinal 209 diffusion can be neglected. This is in agreement with conclusions made by Krylova et al. [8].

210 The data presented in Fig. 2A reveal that introduction of one plug of protein solution and one 211 plug of ketamine and NADPH solution results in partially overlapping zones. It is also apparent 212 that the protein becomes stronger dispersed compared to the low molecular mass components. 213 The same behavior is true with injection of narrower zones. In the case of the seven-plug 214 configuration of Fig. 1B, simulation with GENTRANS predicts a good distribution of the protein 215 throughout the zone structure (Fig. 2B). In contrast, low-molecular-mass reactants become again 216 less dispersed. This difference is due to the much lower diffusion coefficient of a protein (Table 217 1). The lower diffusion coefficient results in a larger Taylor-Aris diffusivity (cf. Eq. 1) and thus 218 enhanced transverse diffusion for the protein. The diffusion value used (Table 1) is typical for a 219 protein [10]. No value was available for CYP3A4 SUPERSOMES. CYP3A4 in the purchased 220 SUPERSOMES is membrane bound and probably has a lower diffusion coefficient than 221 albumin. Based on efforts dealing with CYP2C9 SUPERSOMES, a diffusion coefficient of 1.34 222 $\times 10^{-11}$ m²/s was reported for that system [29]. Using this value instead of 5.94 $\times 10^{-11}$ m²/s 223 resulted in an even broader Aris-Taylor dispersion (red broken line graph in Fig. 2A). For the 224 investigations made in our work, an exact value is not required as spreading of the protein is sufficient for both cases. The diffusion coefficient of NADPH used $(64.21 \times 10^{-11} \text{ m}^2/\text{s})$ 225 226 corresponds to the value given in [29] and was also employed for ketamine. These values may

- not be accurate but they provide sufficient insight into the principle of mixing by transverse
- diffusion when plugs are inserted with a specified velocity. It is important to note that, for small
- 229 molecular mass components, GENTRANS requires the input of a mobility value and calculates
- the diffusion coefficient via the Einstein relation (Table 1).
- 231 The data presented in Fig. 2 suggest that transverse diffusion provides mixing of the reactants in 232 both configurations. Similar results were obtained with the Krylov model (Fig. 3A and 3B), a 233 model which considers differences in the total hydrodynamic transport time intervals of the plugs 234 (time intervals decrease from firstly to lastly injected plug), neglects longitudinal molecular 235 diffusion, and provides profiles of enzyme and reactants only. GENTRANS considers both 236 transverse and longitudinal dispersion, handles reactant and buffer components, and thus provides also distributions of buffer constituents (Fig. 4A), pH (Fig. 2, 4A) and conductivity 237 238 (Fig. 4C). Thus, intrusion of BGE components into the reaction zone is not handled by the 239 Krylov model but is visualized by GENTRANS. In the case of the four-plug configuration with 240 the firstly introduced plug composed of IB only, penetration of the chiral selector into the 241 reaction zone and thus complexation of ketamine which affects the enzymatic reaction is 242 prevented. Furthermore the pH of 7.40 is maintained throughout the reaction mixture (Fig. 2A). 243 For the seven-plug structure, a small part of the reaction mixture is predicted to be affected by 244 the BGE (right hand side of mixture in Fig. 2B). This could easily be prevented by commencing 245 the plug injection procedure with a plug of IB as was the case for the four-plug configuration 246 (Fig. 1A).

247 3.2. Simulation of electrophoretic mixing and buffer changes after plug injection

248 In the previous work with the four-plug structure and employing the NADPH regenerating

- system to promote the enzymatic reaction, short application of voltage (-10 kV for 10 s) just after
- 250 hydrodynamic plug injection followed by the incubation period without applied electric field
- 251 revealed enhanced norketamine formation [25]. Thus, simulation with GENTRANS was
- employed to provide insight into the processes occurring during this period. For the four-plug
- 253 system of Fig. 2A, distributions of enzyme, reactants, buffer components and pH after plug
- 254 injection are shown in Fig. 4A. The impact of application of a constant -1000 V (100 V/cm) for
- 255 16 s without buffer flow is presented in Fig. 4B. Under the influence of the electric field,
- ketamine is predicted to migrate towards the cathode (left hand side in Fig. 4B) and NADPH

257 towards the anode (right hand side). In the same time interval, the distribution of CYP3A4 is 258 predicted to remain almost the same (very small displacement towards the cathode). Application 259 of power is predicted to provide a better mixing of ketamine with the enzyme (in an EMMA type 260 fashion). However, NADPH is migrating in the opposite direction and responsible for a smaller 261 overlap with the other reagents. Thus, the application of power should result in a somewhat 262 smaller reaction mixture of the three components and should not enhance metabolite production. 263 This is in agreement with experimental data obtained with this configuration (Fig. 5B, case I). A 264 typical electropherogram which was obtained with the application of the short power pulse prior 265 to incubation is presented in Fig. 5A. As expected the yield of S-norketamine was larger than 266 that of R-norketamine. This is in agreement with the data reported before [25]. The voltages 267 applied in the experiment and simulation (222.2 V/cm with 0.17 min ramping and 100.0 V/cm 268 with no ramping, respectively) were similar but not equal. Thus, in order to obtain comparable 269 results, a 16 s time interval of voltage application was used in the simulation rather than the 12 s 270 of the experiment.

271 Simulations with GENTRANS provide information about the dispersion and migration of all 272 components, including the buffer constituents (Fig. 4). This is of particular interest in the context 273 of the two buffers used (IB and BGE) which have a different composition and a completely 274 different pH (7.40 vs. 2.51, respectively) and conductivity (2.04 vs. 0.49 S/m, respectively, Fig. 275 4C). The IB provides the right environment for the enzymatic reaction whereas the BGE serves 276 as medium for the enantioselective separation and analysis of the formed metabolites. The buffer 277 change, after hydrodynamic displacement, is located at 5.6 cm of the simulation column (Fig. 278 4A). Upon voltage application, the negatively charged chiral selector becomes removed from the 279 anodic edge of the incubation mixture and a number of concentration boundaries begin to 280 develop. To simplify matters, the chiral selector was modeled as a monovalent strong acid (Table 281 1). Two sharp steady-state moving boundaries that migrate towards the cathode and define the 282 beginning and end of a sodium buffer zone were formed on the anodic side of the potassium 283 based IB (Fig. 4B). These boundaries are formed because the effective electrophoretic mobility 284 of potassium is larger than that of sodium and the mobility of sodium is larger than that of Tris. 285 Sodium originates from the chiral selector in the BGE, migrates towards the cathode and its zone 286 becomes longer with time. Due to Tris in the BGE, a new buffer which contains sodium, Tris and 287 phosphoric acid is formed on the anodic side of the sodium zone. The pH of this buffer is

predicted to be 7.22 and is slightly lower compared to that of the IB (7.40) whereas the

conductivity is significantly smaller (0.77 vs. 2.04 S/m, Fig. 4C). Furthermore, two boundaries

290 migrate towards the anode (Fig. 4B) with one migrating very slowly and representing a large pH

change. A stationary boundary is formed at the location of the initial buffer change (from IB to

BGE, visualized with the buffer distributions depicted in Fig. 4A). These data suggest that with

293 prolonged application of voltage (e.g. > 40 s) incubation would occur in the sodium-Tris-

294 phosphate buffer and not in the foreseen IB.

295 For the data presented in Figs. 2-4, the input data summarized in Table 1 were used. For the 296 enzyme and the two reactants, these data are not accurate but reflect the principle. The effective 297 electrophoretic mobility of the weak base ketamine in IB (pH 7.4, ionic strength of 222 mM) was determined to be 1.00×10^{-8} m²/Vs. The pKa value of ketamine is 7.5 (Table 1) which indicates 298 299 that ketamine in IB is about 50 % protonated. Using Peakmaster and considering the ionic 300 strength, the limiting ionic mobility of ketamine was estimated as 2.35×10^{-8} m²/Vs. This mobility is close to the 2.50×10^{-8} m²/Vs used in the simulation (Table 1). The effective mobility 301 302 of NADPH in IB was found to be $2.98 \times 10^{-8} \text{ m}^2/\text{Vs}$ and NADPH migrated as an anion. NADPH 303 is a multivalent component and supplied as tetra sodium salt. No exact pKa values were 304 available. Thus, in order to mimic the anionic transport in the electric field, NADPH was treated 305 as a monovalent weak acid with a pKa value of 2.0 and a mobility of $2.50 \times 10^{-8} \text{ m}^2/\text{Vs}$ (Table 1). 306 The migration behavior of CYP3A4 SUPERSOMES could not be studied experimentally as the 307 concentration in the purchased reagent was too small. CYP3A4 is claimed by the 308 SUPERSOMES distributor to have a pI value around 8.6 (Dr. M. Gueldenagel, Scientific Support, Corning, email message of November 25, 2014). Thus, the input titration curve for the 309 310 protein was created around this pI value [10]. With this input, the protein is slightly positively 311 charged at pH 7.40. It is not known how realistic this input is as SUPERSOMES comprise also 312 the electron donor partners P450 reductase and cytochrome b5 which are negatively charged 313 proteins at pH 7.4. In SUPERSOMES, all three proteins are membrane bound and there is no 314 information available about the stability of the vesicle in the electric field [30]. These facts, 315 however, are not terribly disturbing as the low diffusion coefficient results in sufficient overlap 316 of the SUPERSOMES with the two reagents during the hydrodynamic injection.

- 317 Experimental results obtained with NADPH-RS as originally used in [25] instead of NADPH are
- 318 presented as case Ia in Fig. 5B. The norketamine production yields obtained for this
- 319 configuration are equal to those observed with NADPH (case I of Fig. 5B). Both approaches
- 320 provide lower norketamine amounts compared to off-line incubation (case off-line of Fig. 5B).
- 321 With the use of NADPH-RS, however, application of voltage resulted in an increased production
- 322 of norketamine enantiomers. This is in agreement with previous data [25] and is different
- 323 compared to case I of Fig. 5B. Reasons for the enhancement of metabolite production upon
- 324 voltage application are not clear. The NADPH-RS reagent mixture comprises NADP⁺, glucose-
- 325 6-phosphate, MgCl₂, glucose-6-phosphate dehydrogenase and sodium citrate. Due to lack of
- 326 proper input data for most of these compounds, the dynamics of such a configuration under
- 327 current flow could not be simulated.
- 328 3.3. Analysis of alternative four-plug arrangements
- 329 In order to further validate the simulation approach used to study diffusional and electrophoretic
- 330 mixing in Sections 3.1. and 3.2., respectively, three alternative four-plug arrangements were
- 331 investigated. These configurations comprised ketamine in the plug with CYP3A4
- 332 SUPERSOMES instead of the plug with NADPH (referred to as case II), NADPH in the
- 333 SUPERSOMES plug instead of the plug with ketamine (case III) and NADPH injected after
- 334 CYP3A4 SUPERSOMES (case IV). They were studied by computer simulation using
- 335 GENTRANS and examined experimentally both without and with application of voltage for a
- 336 short time after hydrodynamic injection of the plugs. Based on simulations, configuration II
- 337 should not provide an improvement compared to the case shown in Fig. 4. With application of
- 338 the electric field, ketamine and NADPH are predicted to migrate in opposite directions such that
- there is less overlap of these reactants and metabolite production became diminished as was
- 340 found for case I (data not shown). Data obtained with NADPH in the enzyme plug are presented
- in Fig. 6A. Electrokinetic mixing is predicted to provide a larger overlap of the three reactants as
- 342 NADPH and ketamine are migrating towards each other (compare solid line graphs with broken
- 343 line graphs of Fig. 6A). Simulation conditions used were the same as those for Fig. 4 such that
- the data can be directly compared. Thus, for case III, application of voltage should result in an
- 345 increased metabolite production. The performed experiments confirmed this prediction (insert
- 346 Fig. 6A). This configuration, however, is not suitable from a practical point of view as NADPH

and CYP3A4 SUPERSOMES should not be combined because of gradual NADPH degradationcaused by CYP reductase.

349 Data obtained for case IV are presented in Figs. 6B and 6C. This configuration should provide 350 the best injection order of reactants for an EMMA based analysis. The first injected plug with IB 351 keeps the BGE separated from the incubation mixture, the second plug poses the substrate 352 ketamine with its positive electrophoretic mobility, the third plug provides CYP3A4 353 SUPERSOMES and the last plug applies NADPH with its negative electrophoretic mobility. 354 Introduction of the plugs is predicted to provide no overlap of NADPH and ketamine (broken 355 line graphs in Fig. 6B) whereas reactant mixing is obtained via electrophoretic transport (solid 356 line graphs in Figs. 6B and 6C). This was experimentally confirmed with no norketamine 357 enantiomers being detected without whereas norketamine enantiomers were monitored after 358 electrokinetic mixing (insert Fig. 6B). The time interval used to study the effect of 359 electrophoretic mixing was twice as long compared to that used for the other configurations. 360 During electrophoretic mixing, buffer changes occur as was discussed above for Fig. 4B. The 361 cationic moving boundary system produced upon current flow behind the leading potassium ion 362 is predicted to migrate faster towards the cathode than ketamine. It thus overtakes ketamine 363 which becomes adjusted to a narrower peak with higher concentration (about 2.08 mM, Fig. 6C). 364 The asterisks in panel B of Fig. 6 mark the locations of the two moving boundaries after 32 s of 365 power application. NADPH on the other hand is predicted to become a broader peak with a 366 concentration maximum around 1.32 mM (Fig. 6C). Part of this process for ketamine is seen in 367 the 32 s distribution presented as solid line graph in Fig. 6B. Complete overlap of both reagents 368 is predicted to occur after 64 s of power application (Fig. 6C). During this merging process at a 369 constant -1.0 kV the current density dropped from -9499.6 A/m² to -8564.7 and -7895.2 A/m² 370 after 32 s and 64 s of power application, respectively. Only one moving boundary migrating 371 towards the cathode would be obtained with the use of sodium instead of potassium in the IB and 372 no cationic migrating boundary (i.e. no buffer change) is predicted with sodium in the IB and no 373 Tris in the BGE (e.g. with 50.14 mM NaOH instead of 50 mM Tris in BGE). Thus, with the 374 latter buffer system, the profiles of the reactants would not deteriorate as is seen in the data of 375 Fig. 6C. These data reveal that simulation provides meaningful data in all four cases.

376 3.4. Experimental results with the seven-plug arrangement

- 377 Norketamine production data obtained with the seven-plug approach are presented in Fig. 7. The
- 378 first configuration assessed was that adopted from the literature [26] for which a typical
- 379 electropherogram is depicted in Fig. 7A. Data of determined peak areas are presented as case I in
- 380 Fig. 7B. As expected the yield of S-norketamine was larger than that of R-norketamine. This is
- in agreement with the data reported before [26]. Furthermore, a 2.5-fold reduction of the plug
- insertion velocity (0.2 psi instead of 0.5 psi) together with 2.5-fold higher injection times such
- that the same plug volumes were injected resulted in a much smaller production of the
- norketamine enantiomers (case I (0.2 psi) of Fig. 7B). These data nicely illustrate that transverse
- diffusional mixing is dependent on the injection velocity of the plugs as is predicted by Eq. 1 and
- also confirms the predictions of the TDLFP model.
- 387 The use of NADPH-RS instead of NADPH in the ketamine solution (case Ia of Fig. 7B)
- 388 provided a somewhat higher norketamine production yield compared to that with NADPH (case
- I of Fig. 7B). Similar data were obtained with off-line incubation (case off-line of Fig. 7B).
- 390 These data reveal that plug injections at 0.5 psi (injection velocity of about 600 µm/s) provide
- 391 good conditions for transverse diffusional reactant mixing in the seven-plug approach. The
- 392 employment of NADPH-RS demonstrated no significant advantage over simple addition of
- 393 NADPH in terms of norketamine production.

4. Concluding remarks

395 The data presented reveal that dynamic computer simulation provides meaningful data for 396 transverse diffusional reactant mixing occurring during plug injection and electrophoretic 397 reactant mixing caused by voltage application after plug insertion. Furthermore, reactant 398 adaptation due to buffer changes which occur upon current flow when a discontinuous buffer 399 system is employed can be investigated and visualized with the same simulator. From the results 400 obtained it is obvious that the injection velocities used experimentally were effective to provide 401 sufficient transverse diffusional reactant mixing during hydrodynamic plug insertion in both plug 402 configurations. Due to the longer plugs employed in the four-plug arrangement, a higher plug 403 injection velocity was required to reach sufficient diffusional mixing. Compared to the seven-404 plug system, the four-plug configuration provides a less defined reaction space. In the latter

405 approach, partial overlap of enzyme and reactants is obtained only. In all cases analyzed, the

406	predicted	mixing of	reactants and	enzyme	could be	validated	experiment	ally	for	the
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- 407 enantioselective N-demethylation of ketamine to norketamine mediated through CYP3A4. No
- 408 fundamental difference between the use of NADPH and NADPH-RS was found. Both cofactor
- 409 sources supply enough reducing equivalents for the enzymatic reaction. The simulation efforts
- 410 conducted in this work illustrate the value of using a dynamic simulator to investigate both
- 411 diffusive and electromigrational aspects of mass transport in on-line systems.

412

413 **Conflict of interests**

- 414 The authors have declared no conflict of interests.
- 415

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