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Kronfeld, Klaus-Peter; Ellinger, Thomas; Köhler, Michael:

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Review Paper

Microfluidically prepared sensor particles for determination of chloride by fluorescence quenching of matrix-embedded lucigenin

Klaus-Peter Kronfeld[1] · Thomas Ellinger[2] · J. Michael Köhler[1]

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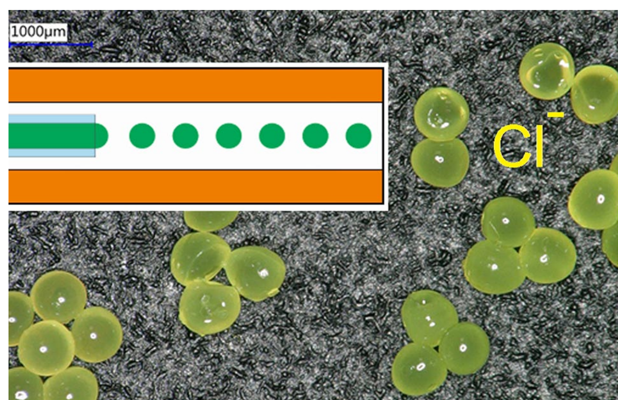
Abstract
Polyacrylamide sensor particles have been synthesized by using a microfluidic arrangement for generation of micro-droplets containing a reaction mixture for forming gel microparticles. The droplets are formed in an inert carrier liquid immiscible with the reaction mixture based on aqueous solutions. Gel particles are formed in situ by photochemical initiation of polymerization inside droplets using an UV-sensitive photoinitiator. In result, water-swellable spheres with submillimetre size are obtained. Those spheres were loaded with N,N′-dimethyl-9,9′-biacridinium dinitrate (lucigenin) as ion selective fluorescence probe for chloride. The particles can be dried, stored and re-swollen. Upon exposure of dried particles to sodium chloride solutions they showed dynamic fluorescence quenching obeying the linear plot of Stern–Volmer-equation between 0 and 130 mM Cl^-. Thus, chloride concentrations up to 50 mM could be measured with appropriate accuracy. The particles allow a fast optical determination of chloride in tiny analyte volumes down to below ten microliters.

Graphic abstract



Keywords Hydrogel particles · Microfluidics · In-situ-polymerization · Fluorescence sensing · Chloride determination · Lucigenin

✉ Klaus-Peter Kronfeld, klaus-peter.kronfeld@tu-ilmenau.de | [1]Department of Physical Chemistry and Microreaction Technology, Institute for Chemistry and Biotechnology, Techn. University Ilmenau, PF 10 05 65, 98684 Ilmenau, Germany. [2]BLINK AG, Winzerlaer Straße 2, 07745 Jena, Germany.

1 Introduction

Fluorescent micro particles attract increasing attention as primary transducers for local chemical sensing [1]. They can be regarded as local sensing elements which convert the information on local concentration of an analyte into a signal which can be contact-free read out by light [2]. The application of this micro sensing elements open the way for determining local concentrations from small volumes as tissue fragments and cells [3] as well as from micro fluidic compartments [4]. The application of particles with immobilized sensor analyte-sensitive dyes instead of dye solutions is motivated by three aspects. At first, the immobilized dye cannot be up-taken by biological cells and, therefore, a toxic effect is avoided. At second, the incorporation of the dye inside the particles can improve its chemical stability and suppress any metabolization effect by physiologically active cells inside the sample. At third, the optical signal is automatically limited to the volume of the particle. This gives the possibility to measure sensor and background signal at the same time, which allows to subtracting any optical background locally, on the one hand. On the other hand, particles open the possibility for simultaneous determination of several components in parallel by spatial separation of signal transduction with different transducer particles distinguished by their shape, size or colour labelling.

Luminescent sensor particles have been developed for the characterization of pH [5] and for oxygen, for example [6]. Despite the application in simple chemical analysis fluorescent particles have also been applied for the sensing of special bioactive molecules as cholesterol [7], for detection of enzyme activity [8] and for the identification of complex biological objects, among them lysosomes [9] and cancer cells [10]. The change of fluorescence quantum yield is applied, for example, in case of some pH-sensitive particles [11]. Oxygen-sensitive optical sensor particles use the fluorescence quenching of triplet dyes for determination of oxygen concentration [12].

Beside pH, oxygen, organic molecules and cells, other small inorganic ions are also important for characterization of cultivation volumes in biotechnology and for chemical analytics. Among them, chloride ions can be proved by the application of lucigenin. The fluorescence of this chromophore is quenched by chloride ions [13].

Here, the application of this dye for the generation of micro sensor particles is reported. These particles should be applicable in aqueous solution. Therefore, a water-swellable matrix is required. It was known from previous experiments that polyacrylamide hydrogel particles meet the requirements for swellability, permeability by inorganic molecules and analyte molecules and for analytical purposes, in general [14]. Such particles can be generated with high homogeneity in size by a microfluidic photochemical synthesis [15]. This type of micro particles can be synthesized by photopolymerization of acrylamide and cross linking in aqueous solution.

The realization of sensor particles was based on the following mechanistic concept: (1) swellable micro gel particles of high size homogeneity are produced by a photochemically induced particle polymerization in microfluidically generated droplets, (2) the sensor dye lucigenin is immobilized in the gel matrix, (3) after embedding the sensor particles inside an chloride-containing aqueous solution the particle swells and takes up the chloride ions together with the aqueous solution, (4) the fluorescence quantum yield is reduced in dependence of the chloride concentration inside the solution resulting in a concentration-dependent optical intensity signal, (5) the signal can be read out contact-free by a Fluorimeter. In the following, it will be shown that this concept is usable for miniaturized chloride sensing.

2 Experimental

2.1 Experimental set-up and procedure

The experimental set-up is adapted from earlier arrangements for the generation of hydrogel and metal/polymer composite particles [16]. A modular microfluidic system consisting on two computer-controlled syringe pumps (Cetoni, Korbußen, Germany), a droplet generation module, an irradiation module, a glass tube for conducting droplets and particles and a particle collector has been applied (Fig. 1). The droplets of monomer mixture are formed by injecting this liquid by a steel capillary into the carrier stream of silicone oil (Fig. 2). Instantly after microfluidic drop generation hydrogel particles run through the irradiation unit undergoing photochemically initiated free radical polymerization. The monomer solution was composed of 450 mg acrylamide:N,N′-methylenebisacrylamide 19:1 in 1,5 mL deionized water with 7.5 mg of the Li-TPO photoinitiator. A 120 watts mercury short arc reflector lamp HXP 120 V (Leistungselektronik Jena) was applied as light-source. Light intensity can be tuned and attenuated by its opto-mechanical dimming unit. Irradiation was performed at room temperature by a 5 mm glass fibre light cable perpendicular to the flow direction at 1/6 of the maximum intensity.

Particles have been separated from the silicone carrier phase by suction over a filter plate. Remaining silicone

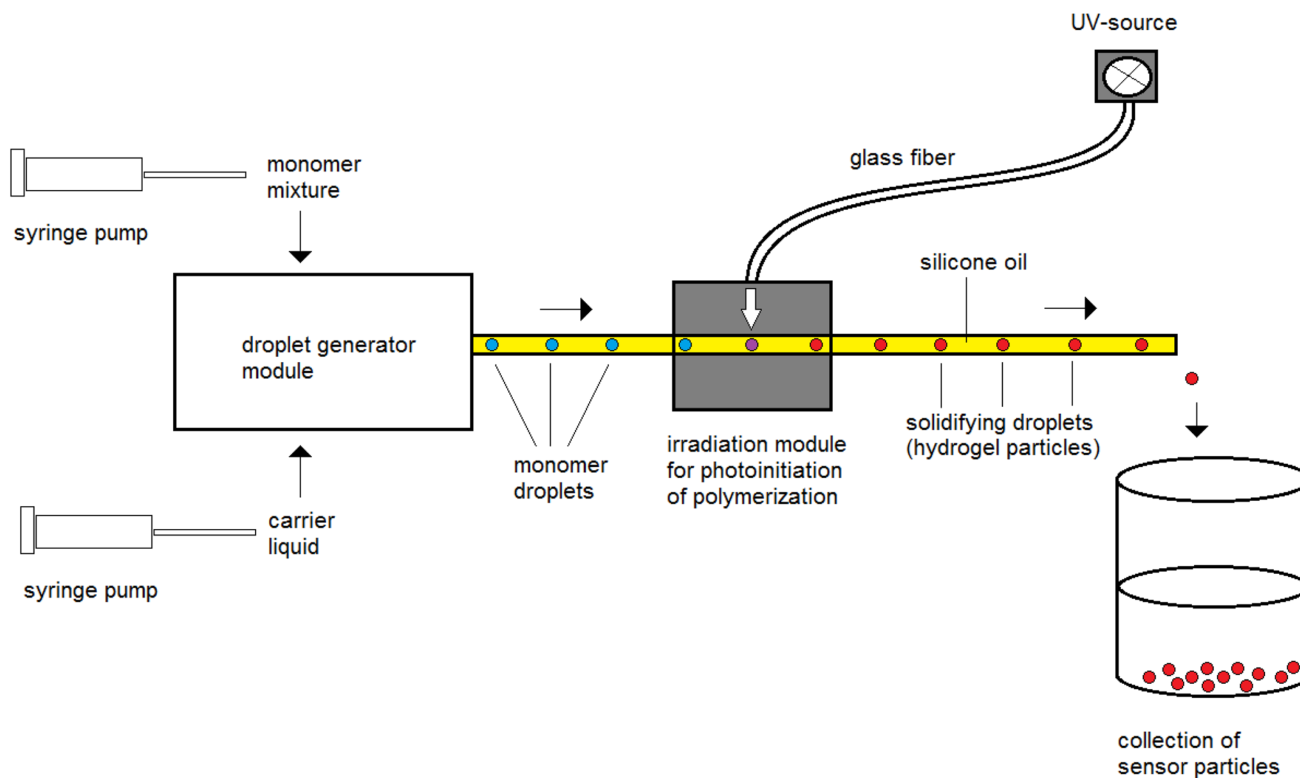


Fig. 1 Experimental set-up for fluidic generation of hydrogel sensor particles

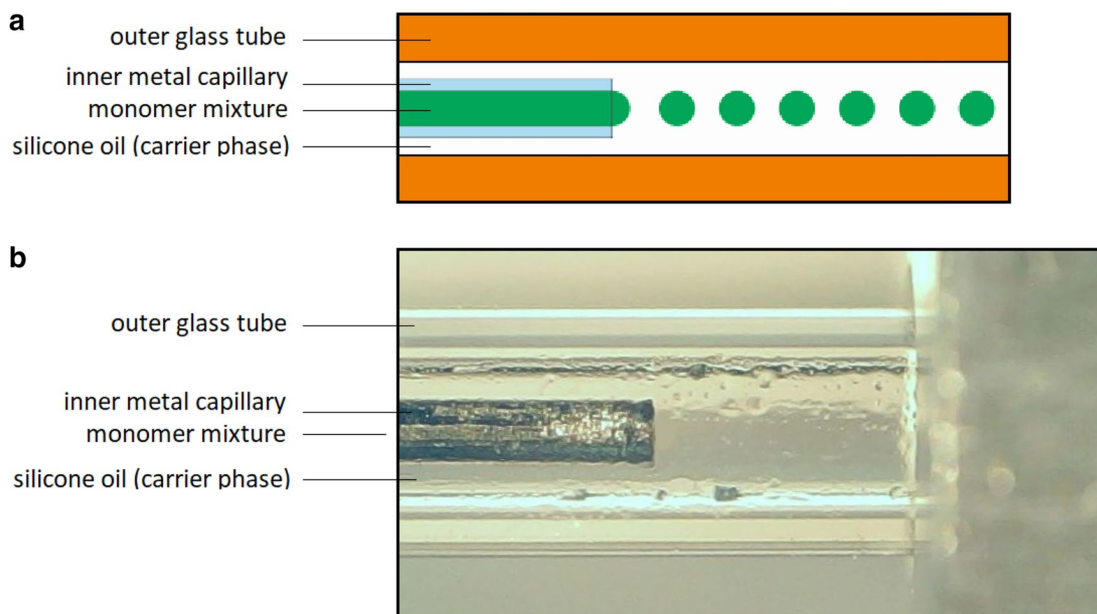


Fig. 2 Capillary arrangement for microfluidic generation of lucigenin-loaded hydrogel particles, **a** schematic, **b** photograph of the capillary-in-tube set-up

oil is removed by washing with n-heptane. Thus cleaned particles have been dried in a desiccator over silicagel at room temperature and 60 mbar for 48–72 h. Then they were immersed in 164 mg/L aqueous lucigenin solution for swelling. After 2–4 h swelling time the particles are again separated and dried as described.

2.2 Spectrometry and microscopy

Absorption spectra of solutions have been recorded by means of a UV–Vis spectrometer Specord 200 (Analytic Jena), uncorrected emission spectra with a fluorimeter JASCO FP-8300. Optical measurement of particles has been carried out by a fluorescence microscope Zeiss Axioplan 2 equipped with a Sony SLT-A37 digital camera. Processing of the measurements of particles sizes and swelling behaviour were executed using standard graphic software IrfanView and in-house application "ChipShopChip" based on LabVIEW (National Instruments) for determination of fluorescence intensities.

The particles can be stored in dried state after washing and loading with lucigenin. The response behaviour of dried particles on water and on chloride ion containing aqueous solutions was monitored by fluorescence microscopy. Therefore, single fluorescing dry particles have been placed in the indentation of a glass slide under the fluorescence microscope. Excitation in the near UV-region (300–395 nm) was applied by means of an optical filter (Zeiss module 1046-281). After adding of liquid, the particle swelling was characterized by taking further photos in time periods of ca. 20 s until the swelling process was almost completed. The photographs allowed to determining the growth rate of particles by the swelling process (change of sizes) and the change in fluorescence intensity simultaneously.

2.3 Chemicals and materials

Silicone oil 500 cSt (Carl Roth) was used as continuous flowing phase inside the micro flow reactor. Acrylamide:N,N′-methylenebisacrylamide 19:1 (for electrophoresis, Fisher Scientific) was used dissolved in deionized water and applied as monomer for particle synthesis. The photoinitiator lithium 2,4,6-trimethylbenzoyl-phenylphosphinate (Li-TPO) was synthesized according to literature [17] from 2,4,6-trimethyl-benzoylphenylphosphinic acid ethyl ester (95%, abcr) and lithium bromide (99%, Carl Roth), 84% yield. A total monomer content of 23% in water was applied for the monomer mixture. Lucigenin (N,N′-dimethyl-9,9′-biacridinium dinitrate) was purchased from TCI; analytical grade n-heptane and sodium chloride were used as supplied.

3 Results and discussions

3.1 Microfluidic preparation of sensor particles

For droplet generation, a flow rate ratio between carrier liquid and monomer mixture of 6.7 was applied. Inside the outer glass-tube (ID 1.5 mm) the continuous phase silicone oil 500 cSt is moving at 200 µL/min flow rate. In the centre of that tube the inner capillary tube is placed introducing the monomer solution at 30 µL/min. It is of metal type (ID 600 µm, OD 900 µm, Fig. 2) and shields scattered light thus preventing undesired polymerization inside the capillary. When leaving the capillary the monomer solution forms spherical droplets co-moving with the silicone oil to pass the subsequent irradiation unit. The droplets are exposed in the spot of UV irradiation during a residence time of about 2.3 s. This time is sufficient for the initiation and propagation of polymerization. The polymerization is running on and completed during the passage of the outlet glass tube. The light-cured hydrogel particles leaving the equipment are finally collected for further treatment—separation, drying, loading with lucigenin—as described above. The finished assay-particles are still swellable having a shelf life of at least 3 months.

3.2 Characterization of particles

The obtained particles have a diameter of ca. 800 µm. After drying, their diameter amounts about 480 µm, corresponding to a volume reduction of 78% (Fig. 3). The microfluidic generation process supplies a narrow size distribution (standard deviation 2–3%).

3.3 Particle swelling

All dried particles show a significant and reproducible swelling after immersion in pure water or in aqueous solution. Upon uptake of liquid, the particle diameter is enlarging nearly to the double size. During this swelling process the concentrations of the fluorophore and of the analyte

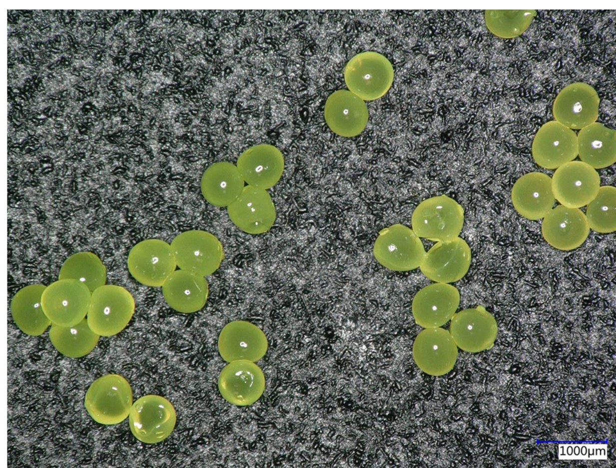


Fig. 3 Optical image of sensor particles after drying (particles somewhat larger, because from a different batch)

are changing as well as the local light absorption and diffusion coefficients inside the particle. Since fluorescence intensity is influenced by these processes, a well-defined narrow particle size distribution both in the dry and in the swollen state is beneficial from a chemometric point of view. The intruding chloride (or even water) forms a firmly shaped diffusional front, which can be seen exactly from the change in fluorescence intensity (Fig. 4).

It is clearly visible that the highest fluorescence intensity is found in the non-swollen core regions of the particles. Even in the case of pure water, the local fluorescence intensity is lowered in the swollen outer matrix of the particle (Fig. 4a). This reduction can mostly be attributed to the volume increase and the related decrease of local concentration of immobilized fluorophores. In case of swelling with chloride-containing solution, the swollen outer part of particle shows a drastic reduction of fluorescence intensity due to the response of lucigenin on the increase of analyte concentration (Fig. 4b).

During swelling the particle diameter enlarges at a more and more decreasing rate, which follows approximately an exponential law as is expected. Contrary, the diffusional front is progressing almost linearly with time to the centre of the particle (Fig. 5). After the luminescent core has resolved the fluorescence intensity remains nearly at a constant value which is dependent on the chloride concentration. During the entire swelling phase the loss of fluorescent dye by diffusion into the surrounding aqueous solution can be neglected, as can also be seen from Fig. 4 since there is no significant light emission outside the particle.

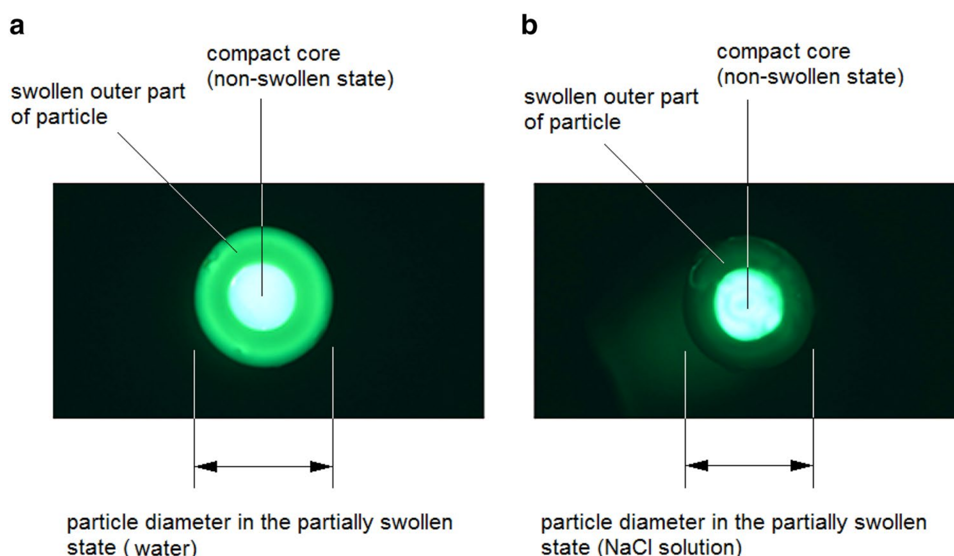


Fig. 4 Optical images of lucigenin-containing PAA sensor particles in a partially swollen state in an aqueous environment: **a** after 70 s of swelling, **b** after 68 s swelling in NaCl solution (130 mM)

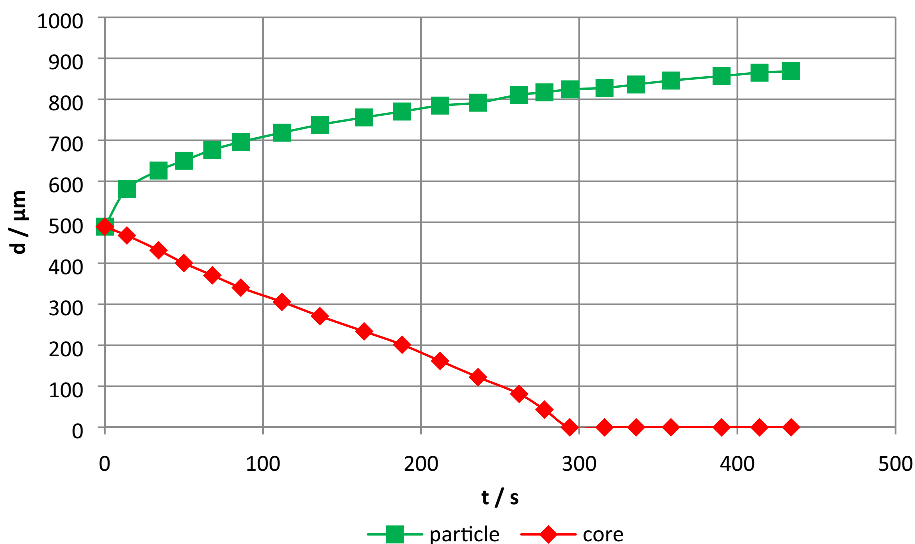


Fig. 5 Swelling behaviour of dried lucigenin-loaded hydrogel particle in aqueous environment (green square outer particle diameter, red diamond diameter of the non-swollen particle core)

The intrusion of water into the core region of particles and the disappearing of the non-swollen core requires about 5 min. After further about 2–3 min the whole swelling process is completed.

Depending on conditions of preparation particle sizes lie in the range of 480 µm in dry and 840 µm in swollen state (Table 1) corresponding to a volume increase by a factor of about 5.4 (increase of 440%). Inside one batch diameters differ 2–3% (standard deviation) of the mean value both dry and swollen. The ratio of swollen to dried particles amounts to 1.7–1.8.

3.4 Determination of chloride by fluorescence quenching of particle-incorporated lucigenin

N,N′-dimethyl-9,9′-biacridinium dinitrate (lucigenin) is a commercially available fluorescence probe for chloride. It has been known for a long time that fluorescence is quenched by halide ions (Cl^-, Br^-, I^-). Neither anions like phosphate and sulphate do interfere [18] with the chloride determination nor pH-changes and oxygen content [19]. As quenching mechanisms heavy atom effect and electron transfer [20] have been discussed. The dynamic quenching process is mathematically described by

$$I = \frac{I_0}{1 + k_q \tau_0^1 [Cl^-]} \quad (1)$$

or in linearized form by the well-known Stern–Volmer-equation:

$$I_0/I = 1 + k_q \tau_0^1 [Cl^-] \quad (2)$$

I_0—fluorescence intensity in the absence of chloride, I—fluorescence intensity at concentration [Cl^-], τ_0^1—fluorescence (singlet) lifetime in the absence of chloride, k_q—bimolecular rate constant, $K_{SV} = k_q{}^* \tau_0^1$—Stern–Volmer-constant

Figure 6 shows fluorescence quenching measured in solution and in hydrogel particles, respectively, with curves fitted to Eq. (1).

Likewise Fig. 7 shows the corresponding Stern–Volmer-plots according to Eq. (2).

For quenching in solution the Stern–Volmer-constants calculated with any of both regressions yields to ca. 300 M^{-1}. Thus, given a singlet lifetime of 20 ns [20] the quenching constant k_q is about $1.5 * 10^{10} M^{-1} s^{-1}$. For hydrogel particles, a constant of 30.9 M^{-1} is calculated with Eq. (1) and 29.7 M^{-1} with Stern–Volmer Eq. (2), respectively. This strongly reduced value can be attributed to the restricted diffusion inside the polyacrylamide gel. The swelling process was always performed with sodium chloride solutions. Therefore, it has to be expected that the chloride concentration related to the aqueous phase is the same inside the gel matrix and in the environment outside the particle. But, the total volume-related chloride

Table 1 Particle sizes of samples from two preparation-batches

Batch	Diameter in µm of lucigenin loaded particles above: dry, below: swollen (at time of measurement)	Mean value	Standard deviation (%)
1	476, 492, 475, 463, 473, 478, 468, 487, 482, 499, 487, 488	481	2.2
	833, 859, 814, 810, 821, 818, 805, 818, 828, 830, 835, 838	826	1.8
2	480, 494, 476, 480, 521, 473, 478, 485, 466, 492	484	3.2
	848, 871, 838, 871, 898, 836, 847, 840, 827, 838	851	2.6

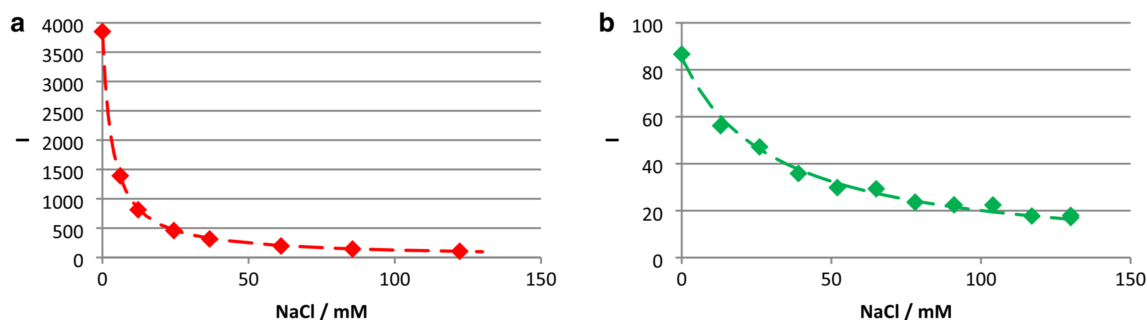


Fig. 6 Comparison of fluorescence quenching of lucigenin in aqueous NaCl-solution (**a**) and of lucigenin-containing PAA sensor particles (**b**), regression curves according to Eq. (1), **a** λex 430 nm, λmeas 489 nm, [Lucigenin] = $1.93 \cdot 10^{-5}$ M, **b** measured intensity green colour channel instantly after main swelling process, [Lucigenin] ≈ $2.6 \cdot 10^{-4}$ M

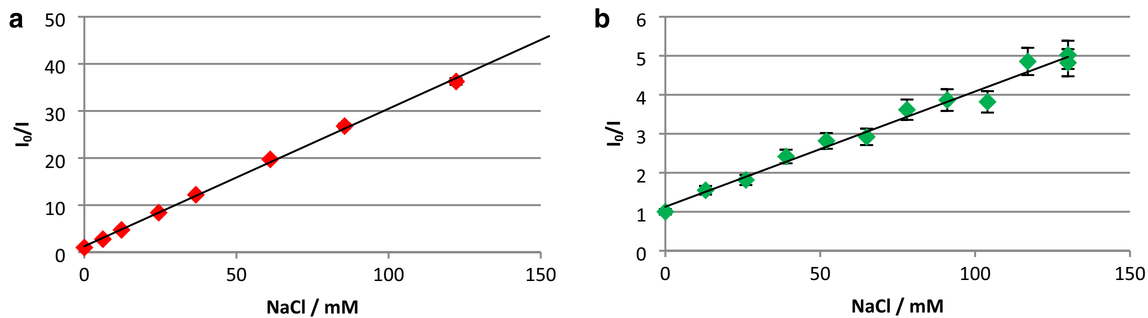


Fig. 7 Stern–Volmer plot for the dependence of fluorescence quenching of lucigenin in aqueous NaCl-solution (**a**) and of lucigenin-containing PAA sensor particles (**b**), **a** λex 430 nm, λmeas 489 nm, [Lucigenin] = $1.93 \cdot 10^{-5}$ M, **b** measured intensity green colour channel instantly after main swelling process, [Lucigenin] ≈ $2.6 \cdot 10^{-4}$ M

Table 2 Errors observed at Cl^-—determination using lucigenin loaded hydrogel particles

Intensity	$[Cl^-]$	$[Cl^-]_{calc}$	error	
a. u.	mM	mM	abs.	rel. (%)
86.7	***0***	***0.0***		
56.2	13	17.3	4.3	32.9
47.2	26	26.7	0.7	2.7
35.8	39	45.2	6.2	15.9
29.8	52	60.7	8.7	16.7
29.3	65	62.3	− 2.7	− 4.1
23.6	78	85.0	7.0	9.0
22.5	***91***	***91.0***		

concentration in the swollen particle has to be corrected by the volume which is occupied by the polyacrylamide matrix material. Therefore, the real analyte concentration in the particle is somewhat lower than the original one in the aqueous solution as a result of the volume contribution of the gel-forming polymer. When the particle swells to the 1.72-fold size (Table 1) the volume increases to the 5.1-fold resulting in a concentration value of 80% of chloride concentration inside the particle ((5.1−1)/5.1 = 0.80) in comparison with the pure aqueous solution and increase of K_{SV} by 25%.

As can be seen from the measurements of Fig. 6b chloride determination up to ca. 50 mM is feasible using the lucigenin fluorescence probe. Higher concentrations may be measured by preliminary diluting of the analyte. Measurements also demonstrate that Eqs. (1) and (2) are valid in good approximation for describing the intensity-concentration-relationship. However, different particle batches will certainly show individual Stern–Volmer-constants K_{SV}. Therefore, a calibration for every batch of particles is usually inevitable. This can be done by a simple 2-point calibration, e.g. at 0 mM and 100 mM. It should be expected, that different analyte matrices might modify the fluorescence quantum yield in general. This effect has to be reconsidered by a suited calibration, too. Table 2 shows absolute and relative errors for the measurements from Fig. 6b, with two pairs of values (the bold ones) taken for calibration.

It should be pointed out that these rather high relative errors are due to single measurements with one different particle for each chloride concentration. For practical application it is recommendable to use a set of particles for each measurement in order to reduce random error. Favourably, the number of particles could be enhanced by reducing their size. It is important that measurements are always taken at the same stage within the swelling process at a point after the diffusion front has reached the particle centre.

4 Conclusions

The investigations showed that the principle of chemical sensing by polyacrylamide hydrogel particles can be applied for the determination of chloride ions in aqueous solutions. The chloride-sensitive fluorescence dye lucigenin could be introduced into the polyacrylamide matrix of the hydrogel particles. Therefore, the continuous flow synthesis of micro particles by microfluidic generation of droplets of monomer mixture in an inert carrier and the subsequent polymerization by an UV-induced radical chain reaction had applied successfully. The procedure supplies particles with high homogeneity in size and fluorescence activity.

The low diameter of sensor particles allows measurements with sample volumes down to about 10 µL. The sensor particles can be used in water or aqueous solutions directly in dried form. The response time in analytic measurements is given by the required swelling time, which is about 5–7 min. The sensing response of particles is well reproducible and reversible. The dependence

of the fluorescence signal from single sensor particles on the chloride ion concentration follows the Stern–Volmer-equation with high accuracy. An inverse linear response of the fluorescence intensity on the chloride concentration was found in the concentration range between 0 and 130 mM sodium chloride. The results show that the sensor particles are well suited for the determination of chloride in the physiologically relevant concentration range.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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