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Fluorescent gel particles in the nanometer range for detection of metabolites in living cells.

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1. Introduction

Particles with embedded fluorescent dyes have potential as nanosensors for the detection of spatially and time resolved measurements of metabolite concentrations in living cells[1,2]. The quantification of the potential analyte is based on ratiometric detection of fluorescence from two dyes. The dyes are chosen such that the fluorescence of one dye is a function of an analyte concentration whereas the fluorescence of the other dye is independent of variations in the medium.

Both acrylamide based particles synthesized in water in oil microemulsions and silica based particles synthesized by variations of sol-gel methods are investigated. Water in oil microemulsions are pseudo 3 component systems (oil/water/surfactant), which in the general case can be found in a variety of phase states depending on the composition and the identity of the components. For the synthesis of particles the interesting phase state is inverse micelles (water in oil micelles). We have investigated the influence of the acrylamide concentration on the phase boundary between inverse micelles and macroscopic phase separation.

In order for the nanosensor to work in practice it is a requirement that the dyes stay in the particles for sufficiently long to perform a measurement. The leaching of fluorescent dyes from particles have been studied and prevented by covalently linking dyes to the particles.

Methods for characterizing particles include size exclusion chromatography and photon correlation spectroscopy, and scanning electron microscopy.

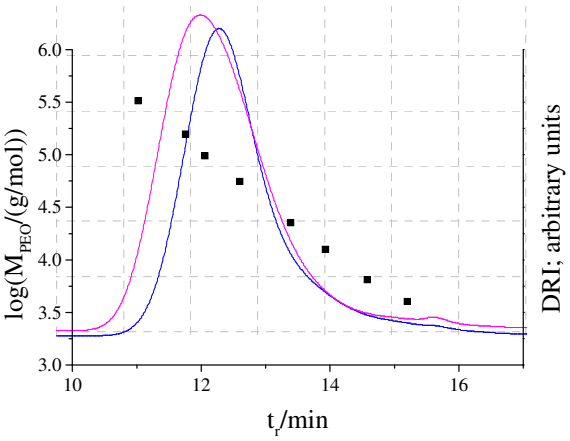


Figure 1. SEC of acrylamide gel particles. Pink and blue curves are two different samples. ■ are calibration with polyethylenoxide standards.

2. Materials and Methods

Brij30 (polyoxyethylene 4 lauryl ether) was obtained from SIGMA. AOT (sodium bis-2-ethylhexylsulphosuccinate), 98%; acrylamide, 99+%, electrophoresis grade; hexane, 95+%, HPLC-grade; N,N´-methylenebisacrylamide, 99+%, electrophoresis grade; fluorescein isothiocyanate 90+%, rhodamine B isothiocyanate; fluoresceinamine; tetraethyl orthosilicate 98+% (3-aminopropyl)-triethoxysilane 99+% were obtained from Aldrich. The buffer was 10 mM sodium phosphate, pH 7.2. Acrylamine, acryloyl chloride and N,N-methylenebis(acrylamide) were obtained from Fluka.

Microemulsions were prepared by adding the aqueous components to a solution of surfactants in hexane. The aqueous component are buffer with or with out dissolved acrylamide and N,N´-methylenebisacrylamide. For preparing particles initiator dissolved in water was added[1]. For studying phase boundaries the aqueous components was titrated with a solution of surfactants in hexane until a microemulsion was obtained or a microemulsion was titrated with the aqueous components until phase separation occurred. Silica based particles were prepared following Santra[3].

Size exclusion chromatography was performed with 25mM NaCl in water as eluent and using a 300×7.8mm^2 PW4000 and PW2500 gel column set from TSK. Differential refractive index and 90° light scattering detection were employed.

Hydrodynamic particle diameters of micelles in microemulsion were determined by dynamic light scattering (DLS) using a BI-200SM from Brookhaven Instruments: this incorporates a 632.8 nm HeNe laser operated at a fixed scattering angle of 90°. Temperature was kept constant with a thermostatted water bath operating at 30°C.

Protoplasts are plant cells without cell wall: The cell walls of 5-day-old tobacco BY2 cells were digested by treatment with 0.1% Pectolyase Y-23, 0.2% Driselase and 1 % Cellulase “Onozuka RS”. Protoplasts were liberated after incubation at 37°C for 2 hours and recovered from the solution by centrifugation. Purification was performed by centrifugation in a Ficoll density gradient followed by several washings in a high osmotic buffer.

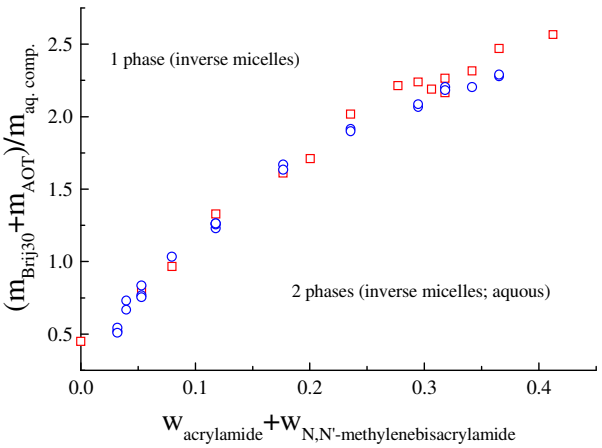


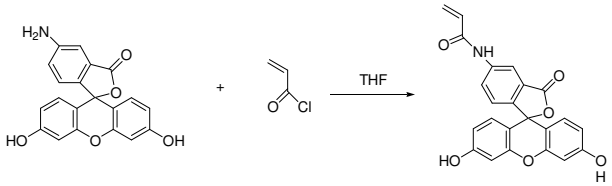
Figure 2. Phase boundary between microemulsion and two-phase region at room temperature. □ Mixing aqueous components in microemulsion until phase separation. ○ Mixing oil and surfactant into the aqueous components until homogenisation. The ratio $\mu=m_{AOT}/m_{brii30}$ was 1.94.

Nanosensors were delivered to protoplasts by a gene-gun. A monolayer of protoplasts in a Petri dish was bombarded with nanosensors in a BioRad PDS-1000 gene gun. A vacuum of 0.5 Bar was applied to the system, and a firing pressure of 50 Bar was used to release nanosensors from the carrier membrane. Protoplasts were subsequently washed several times to remove extracellular nanosensors.

3. Results and Discussion.

Prepared nanoparticles were characterized with a variety of methods. Dynamic light scattering allows the comparison of the micelle size before and after polymerization as well as characterization of the isolated particle. SEC chromatograms of nanoparticles are given in Figure 1. Size measurement using SEC basing the size measure on known relationships between molar mass and radius of gyration for the standards (PEO)[4], using dynamic light scattering where the hydrodynamic size is obtained through the Stokes-Einstein relation, or with small-angle X-ray scattering yield similar results, which in the particular example are particle diameter in the 20 nm range.

The use of inverse micelles as nanoreactor is based on the implicit assumption that the system can be understood as a pseudo 3-component system. The 3 components are the oil, the mixture of surfactants and the aqueous components, which consist of water, buffer and monomers. During the polymerization the aqueous component is changed to water, buffer and polymer. It is assumed that the microemulsion phase behaviour independent of the changes brought about by the polymerization. Furthermore the system should be independent of small variations of the reaction conditions. In order to test this assumption the amount of surfactant



Scheme 1

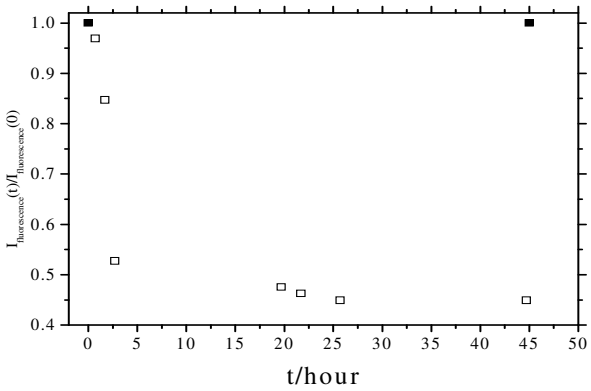


Figure 3. Leaching of physically embedded (■) and covalently linked (□) fluorophores (fluoresceinamine) from polyacrylamide particles.

necessary to form a microemulsion was measured as a function of the concentration of monomer in the water component (Figure 2).

The amount of water that can be incorporated in the microemulsion as inverse micelles depends on the minimum curvature the interfacial layer can sustain. If the volume of the aqueous components is $V_{aq.comp.}$ and the surface area of the spherical micelles is $A_{aq.comp.}$ then the radius of the micelles, r_{mic}, will be given by:

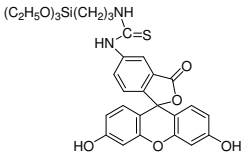
$$r_{mic} = \frac{(\mu+1)}{\theta(\frac{\mu}{M_{AOT}}a_{AOT}+\frac{1}{M_{Brij30}}a_{Brij30})\rho_{aq.comp.}}$$

where $\theta=(m_{AOT}+m_{Brij30})/m_{aq.comp}$, m_{AOT}, m_{Brij30}, $m_{aq.comp}$, are mass of AOT, Brij30 and the aqueous components (the combined mass of water, buffer and monomers), M_{AOT}, M_{Brij30} and a_{AOT} , a_{Brij30} are the molar masses and molar head-group areas of the surfactants, and $\rho_{aq.comp.}$is the density of the aqueous components. Since the head-group areas of surfactant are week function of the condition under which they are used the function $f(\overline{a_i}) = (\mu a_{AOT}/M_{AOT} + a_{Brij30}/M_{Brij30})\rho_{aq.comp.}$ is essentially constant. Thus $r_{mic} \sim 1/\theta f(\overline{a_i})$ and Figure 2 shows that the amount of aqueous component that the microemulsion can contain is substantially reduced when monomer is present - the maximum stable micelle size is reduced. Alternatively the surface area is reduced which conceivably can only happen if surfactant is removed from the water/oil interface.

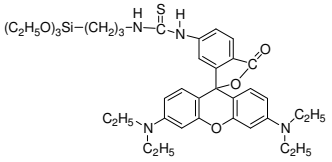
Stable embedding of fluorophores in gel particles can be accomplished either through physical retention of the embedded molecules or through covalent bonding. Covalent attachment is preferable but also more cumbersome since it requires synthesis of fluorophores functionalized with a reactive group capable of reacting with the polymer forming the gel particle either during or after polymerization – see scheme 1. Figure 3 shows a typical difference in leaching properties that result from the two strategies. The covalently bonded fluorophores do not leach whereas a substantial fraction of the physically embedded fluorophores is quickly removed from the particle. However, a significant fraction of the fluorophore is retained in the particle at least for substantial period of time.

The ability of the nanoparticles to report a physical measurement have been tested by recording the re-

sponse to pH of a silica based nanoparticles where a pH-sensitive dye and a reference dye have been covalently attached.



APS-FITC
pH indicator dye



APS-Rhodamine B ITC
pH reference dye

In Figure 4 the ratio of the fluorescence intensity of the two dyes is shown. The pH response is as one would expect.

Fluorescent labelled acrylamide based particles were delivered to protoplasts (plant cells without cell wall) by bombardment with dry particles delivered from a gene-gun. After suitably washing the protoplasts confocal fluorescence microscopy shows – Figure 5 - that the particles can be delivered to cells and retain their activity.

4. Conclusion.

Fluorophores have been embedded in nanometer sized particles and delivered to protoplasts

5. Acknowledegement.

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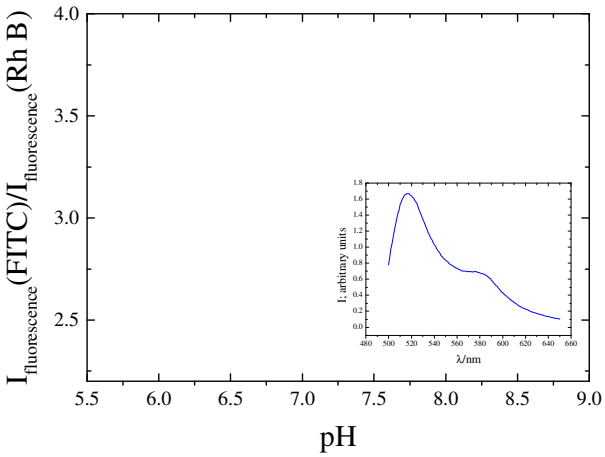

Figure 4. Ratio of fluorescence intensity between FITC (500-540 nm) and Rhodamine B (570-600 nm) versus pH. The inset shown the fluorescence spectrum at pH=6.71. Excitation wavelength was 488 nm.

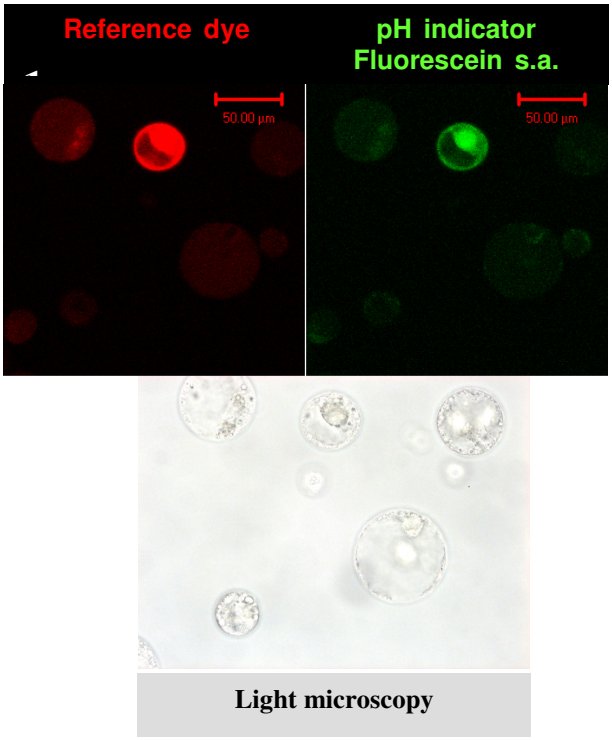

Figure 5. Nanosensors in protoplasts. Leica LSM5 Pascal scanning confocal microscope system equipped with a C-Apochomat 40x/1.2W corr. objective. Fluorescein sulphonic acid and sulforhodamine 101 were excited by the 488 nm Ar and 543 nm He/Ne laser lines, respectively. Emission was collected through a BP 505-530 and a LP 560 filters, respectively.