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Iga WASIAK[1], Suzanne FERY-FORGUES[2], Tomasz CIACH[1]

e-mail: i.wasiak@ichip.pw.edu.pl

[1] Zakład Biotechnologii i Inżynierii Bioprocesowej, Wydział Inżynierii Chemicznej i Procesowej, Politechnika Warszawska, Warszawa

[2] CNRS, ITAV-USR 3505, Advanced Technology Institute in Life Sciences, Toulouse, France

Precipitation of fluorescent micro- and nano- crystals in polysaccharide shell

Introduction

Investigation of many fundamental processes in life sciences requires direct tools for the fast, sensitive, reliable, and reproducible detection of the interaction of biomolecules with one to another, and with various molecular or ionic species. One of the most popular and best-suited methods is the usage of photoluminescence or fluorescence techniques in conjugation with functional dyes and labels. Despite the many advantages of these methods they have significant disadvantages, therefore research on improved dyes are still carrying out.

Numerous scientific research on micro- and nanocrystals are focusing on inorganic compounds such as a metal and a semiconductor materials. Proposed reprecipitation method is bottom-up type with organic crystals obtained as water dispersion. Crystals produced this way exhibit several unique properties, especially for life science labeling. They offer maximal concentration of dye molecules in the minimum volume with much better chemical and biochemical stability than dispersed dyes. The crystal arrangement allows an excellent control of the optical properties in the solid state [*Fery-Forgues, 2013*]. It has been shown that organic crystals can be used for labeling cell cultures before observation with a fluorescence microscope [Baba et al., 2009] and for phototherapy in mice [*Baba et al., 2007*]. Interestingly, they are rather stable within the living cell, where they can stay for a long period of time without leaking out to culture media, thus enabling long-term cell tracking as well as monitoring of a whole biological event. Because of their outstanding intracellular retention, these crystals do not contaminate cells of a different type in a co-culture system, hence permitting growth tracking of a specific cell line [*Yu et al., 2011*]. In vivo, organic dye crystals have been used effectively as functional biomarkers for the identification of increased vascular leakiness, typical of cancer and inflammation [*Sandanaraj et al., 2010*].

Materials and methods

The fluorescent dye 4-n-octyloamino-7-nitrobenz-2-oxa-1,3-diazole (NBD) and berberine palmitate salt (BER-PAL) were received from European grant partner *Laboratory IMRCP* (Toulouse France). Highly pure demineralized water, prepared by *Milli Q* apparatus (*Merck-Millipore*) and absolute ethanol (*Carlo Erba Reagents*) were used as solvents. Dextran (70 kDa) was received from *Nobius* (Jabłonna) and dodecylamine was purchased from *Sigma Aldrich*.

Process of crystals formation was controlled by recording UV/Vis spectra (*Helios γ, Thermo Electron*). The size and shape of crystals were observed with scanning electron microscopy (*FEI*) and fluorescent microscopy (*Nikon eclipse Ti*). To prepare the samples for the scanning electron microscopy observation, a droplet of microcrystals suspension was deposited on a carbon grid. Then the samples were allowed to dry for 24 h at room temperature. Florescent microscopic investigations were carried out by observation from a droplet of the suspension deposited between slide and cover glass after reprecipitation was complete. For both dye excitation wavelength was 450÷490 nm and emission 505÷530 nm. To determine the microcrystals size 150 microcrystals from 5 different observations were calculated.

In the experiment two reprecipitation methods were examined. First reprecipitation method (M1) was carried out according to the method received from the French partner (also described in articles [*Abyan et al., 2006*]). Briefly dye was dissolved in absolute ethanol with final concentration $1 \cdot 10^{-3}$ M, than 40 µl of this solution is rapidly injected to 1.96 ml of water solution. Time necessary to completion process mention in literature is 1 h for NBD and 10 min for BER-PAL. To be sure if the conditions of the reaction are similar and the reaction time was proper, process was monitored by collecting UV/Vis spectra every 10 min. Idea of this process is shown in the Fig. 1a.

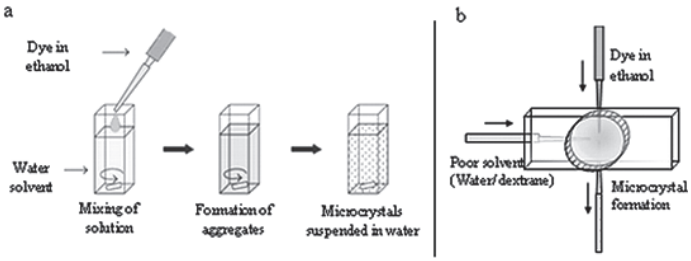

Fig. 1. Reprecipitation method a) idea of method M1 b) structure of microfluidic device for method M2

Second method (M2) for crystals reprecipitation was made in microfluidic devices prepared in our laboratory. Schema of examined system is illustrated on Fig. 1b. Flow ratio of dye solution was 1 ml/h (concentration $1 \cdot 10^{-3}$ M) and water 49 ml/h. This flow rations were calculated from the first method where the volume of the water is 49 times bigger than dye solution. Solution was collected in glass and was left stirred, on the time mentioned in method M1. Moreover, for both method (M1 and M2), experiments where the pure water was change to the 5% water solution of dextran with 1% dodecylamine also were carried out. This change was dedicated by the next step of experiments, which is the formation of polysaccharide shells.

Results

During the reaction was carried out following observations were made. In method M1 after mixing NBD dye solution with water, solution became yellow. Then the solution started to lose its color as the organic compound precipitated. After about 1 h solution became discolored, and visible red agglomerated crystals were deposited on the stirrer and on the wall of the glass. When precipitation was made in water-dextran-dodecylamine solution agglomerate deposition was less observed and whole solution has a red color. In method M2 colors of the solution were similar – water was colorless and water-dextran–dodecylamine was red, but in water agglomerate deposition was no observed. Similar observations were made for the BER-PAL dye. After mixing the dye solution with water, the mixture rapidly became cloudy. In method M1 after 30 min solution become colorless and the agglomerates of the crystals, like small needles, were observed near stirrer. Observation for dextran solution and second method of reprecipitation were as for NBD dye.

The process of crystals formation was monitored by UV/Vis absorption spectroscopy (Fig. 2). After mixing, the spectra displayed two ma-

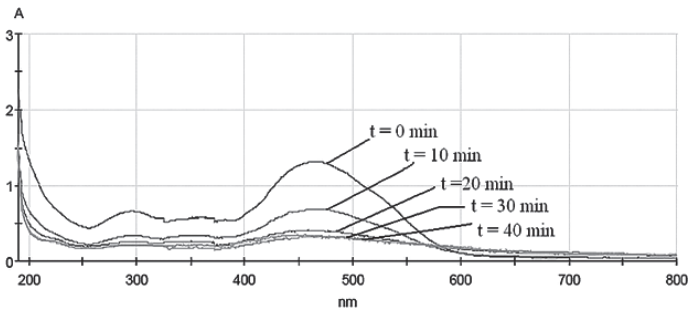

Fig. 2. Evolution of the UV/Vis absorption spectrum of dye during the reprecipitation process, measured every 10 min.

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xima, which indicated that dye is surrounded by the ethanol [*Abyan et al., 2006*]. As the reprecipitation continued, position of the spectrum intensity decreases and they became almost completely reduced by the time the process had been completed. The change in the absorbance is caused by the ethanol progressive dispersion in the aqueous. Time when the reprecipitation process is achieved is calculated when no further change of the absorbance spectra is detected. For the NBD dye time was calculated like reported in literature [*Bertorelle et al., 2003*] around 50 min, while for BER-PAL it takes 30 min and was 3 time longer that reported in literature [*Chahine et al., 2011*].

Comparison of samples SEM imagines, drawn to the conclusion that the use of the system from method M2 reduces the number of new aggregates (Fig. 3a,b). Moreover, use of water dextran-dodecylamine solution eliminated them completely (Fig. 3c,d). The exact measurement of crystal size was not possible; however, obtained images lead to the conclusion that the crystals obtained by method M1 with dextran-dodecylamine solution are smaller than those from method M2 with the same solution. Analysis of the NBD dye crystals obtained from the microfluidic system (M2) shows that they have the shape of sticks (Fig. 3c) and BER-PAL needle shape which is in accordance with previously mentioned literature data.

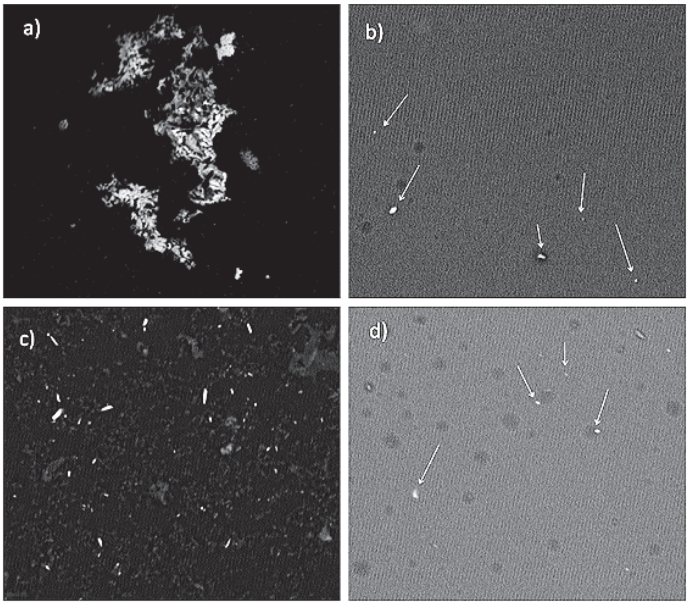

Fig. 3. Scanning electron microscopy images of NBD microcrystals reprecipitated from method: a) M1 with water b) M1 with dextran-dodecylamine solution c) M2 with water and d) M2 with dextran-dodecylamine solution; magnification 5000×

Fluorescent microscopic observation confirmed that, NBD crystals have a shape of stick, while BER-PAL needle. For the dye NBD the following observation and size results were received (Fig. 5). Crystals obtained with reprecipitation with method M1 have longer side size between 2÷7 μm, with big aggregates (15 μm) when were precipitated in water, and 1÷3 μm when were precipitated in water dextran-dodecylamine solution. With method M2 we received crystals with size 3÷6 μm for water and 4÷5 μm for the dextran-dodecylamine solution. No difference in shape of obtained microcrystals was observed.

When the suspension of the dye NBD was left for a couple of hours, during microscope observations a regular sticks were observed. Like we expected, size of crystals became bigger, especially for those received from microfluidic system, and achieved between 8÷10 μm. Crystals precipitated in water achieved two population, small crystals less than 5 μm and big aggregates with size bigger than 20 μm, which were built from smaller crystals. Presence of polysaccharide influence the shape, characteristic notch on their little side can be observed which is in accordance with literature data [*Abyan et al., 2009*]. After this time, in pure water, number of visible red aggregates on the glass wall and stirrer grown, while such observation was no noticeable in dextran-dodecylamine solution. These results were also confirmed for BER-PAL dye.

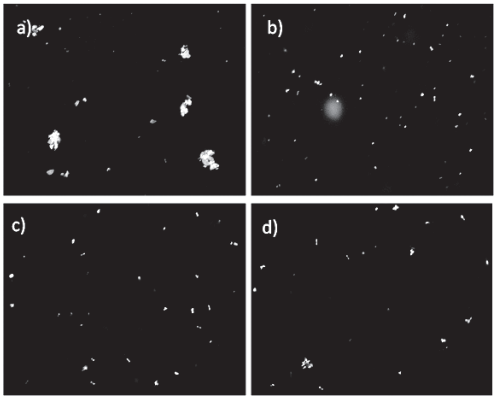

Fig. 4. Florescence microscopy images of NBD crystals after 1h of reprecipitation a) M1 with water b) M1 with dextran-dodecylamine solution c) M2 with water and d) M2 with dextran-dodecylamine solution; magnification 60×

Conclusions

Both reprecipitation methods allow obtaining microcrystals with a related size range. Two methods of observation give corresponding results. First reprecipitation method (M1) with assumed parameters of the second method (M2) enables obtaining of smaller crystals, however, they size distribution is broader.

The use of microfluidic system allows receiving a more similar size distribution. Microfluidic system also reduces the number of observed aggregates.

To receive smaller crystals, it is necessary to further select the precipitation parameters, especially the flow ratio.

The use of a dextran - dodecylamine solution reduces the size of the microcrystals obtained by M1 method. In a case of the precipitation method with microfluidic system we have observed different effect, the size of the microcrystals was not reduced, but its distribution was narrower.

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