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Zirconia nanocrystals as submicron level biological label

K Smits[1], J Liepins[2,3], M Gavare[2], A Patmalnieks[2], A Gruduls[1] and D Jankovica[1]

[1] Institute of Solid State Physics, University of Latvia, 8 Kengaraga Str., Riga, Latvia LV-1048
[2] Institute of Microbiology and Biotechnology, University of Latvia, Kronvalda Blvd. 4, Riga, Latvia LV-1586

E-mail: janis.liepins@lu.lv

Abstract. Inorganic nanocrystals are of increasing interest for their usage in biology and pharmacology research. Our interest was to justify ZrO_2 nanocrystal usage as submicron level biological label in baker's yeast *Saccharomyces cerevisia* culture. For the first time (to our knowledge) images with sub micro up-conversion luminescent particles in biologic media were made. A set of undoped as well as Er and Yb doped ZrO_2 samples at different concentrations were prepared by sol-gel method. The up-conversion luminescence for free standing and for nanocrystals with baker's yeast cells was studied and the differences in up-conversion luminescence spectra were analyzed. In *vivo* toxic effects of ZrO_2 nanocrystals were tested by co-cultivation with baker's yeast.

1. Introduction

A great variety of measurement methods are used currently in biology and pharmacology; however, the demand for more measurement possibilities is continuous. Many of the new methods include usage of inorganic nanocrystals [1,2].

Zirconia nanocrystals are promising candidate for biosensing applications due to their chemical stability and mechanical and optical properties [2,3]. On one hand, zirconia optical properties are oxygen sensitive [4] while on the other hand, it is possible to use up-conversion luminescence properties for temperature measurement [5]. Due to infrared excitation, up-conversion luminescence applications in biology have several advantages over the "classical" one-photon Stokes process, including: lack of autofluorscence, larger penetration depth and less harmful for cells [6, 7].

Information on the nanostructured ZrO_2 effects on organisms *in vivo* is scarce. Thus far, ZrO_2 has been proposed in medical implants, assuming that the material is biologically inert [8]. In a comparable study of metal oxide nanocrystal toxicity in mammalian cell lines (human mesothelioma and rodent fibroblasts), ZrO_2 revealed medium toxicity [9, 10]. Thus, we conclude that zirconia nanoparticle effects on live organisms are loosely covered, especially regarding traditional eukaryotic model organisms such as baker's yeast *Saccharomyces cerevisiae*.

In the work presented here, we synthesized zirconia nanocrystals, measured up-conversion luminescence from nanocrystals in biological media, and checked the impact of different ZrO_2 nanocrystal concentrations on baker's yeast *S. cerevisiae* growth. An attempt to simultaneous submicro scale imaging of *S. cerevisiae* with ZrO_2 nanocrystals was also made.

[3] To whom any correspondence should be addressed

2. Materials and methods

2.1. Zirconia nanocrystal synthesis

The ZrO2 nanocrystals with 1 mol% Er2O3 and 2 mol% Yb2O3 were prepared by the sol-gel combustion technique [11, 12]. After synthesis, the samples were calcinated at 900ºC for 2 hours. Phase composition of the prepared sample was determined by X-ray diffraction analysis (XRD) (D8 Advance, Bruker AXS). The determined grain size was 40 nm. The crystallite size was calculated from the broadening of diffraction maxima using Topas 3 software (Bruker AXS). ZrO2 nanocrystal transmission electron microscopy (TEM) was done by Jeol JEM-1011 instrument.

2.2. Yeast strains and growth conditions

The *S. cerevisiae* haploid reference strain BY4741 (EUROSCARF, Germany) was used for toxicity studies. The yeasts were grown in the presence (1, 2, 5 or 20 mM) or absence of ZrO_2. Cells were cultivated as reported elsewhere [13]: in media containing 2% **P**eptone, 1% **Y**east extract, 2% **D**extrose (further abbreviated as YPD), +30ºC, on orbital shaker with agitation rate 180 revolutions per minute. Yeasts were inoculated at OD600=0.2 and grown for 22 hours. 2 ml samples for biomass measurements were harvested and frozen for further analyses.

2.3. Biomass concentration and glucose measurements

The yeast growth was measured spectrophotometrically at 600 nm (**o**ptical **d**ensity at **600** nm, further abbreviated as OD600); one absorbance unit of OD600 equals approx. 2×10^7 yeast cells/ ml. OD600 measurements were done on the Ultraspec 2100pro (GE Healthcare Life Sciences) instrument using empty YPD broth as a reference.

Since ZrO_2 nanocrystals increase the absorbance of cell suspension at 600 nm, thus giving erroneous estimation of cell growth dynamics, an alternative Fourier transform infrared spectroscopy (FT-IR)s based method for biomass measurements for samples containing ZrO_2 was developed. FT-IR analysis was carried out using the HTS-XT microplate reader (Bruker, Germany) over the range 4000-600 cm^{-1} and data processed using OPUS 6.5 software. The spectral region specific for lipids (3002-2797 cm^{-1}) [14] was chosen to linearly correlate OD600 curve. No absorption of ZrO_2 particles over the chosen wavelength region was observed.

Glucose concentration was estimated by sulphuric acid anthrone method [15].

2.4. Luminescence measurements

The luminescence measurements were prepared using the L975P1WJ 975 nm laser diode (Thorlabs) as the excitation source. The luminescence spectra were recorded using the Andor Shamrock B-303i spectrograph equipped with a CCD camera (Andor DU-401A-BV) at exit port. Nanocrystal luminescence imaging with baker's yeast cells was done with a modified Carl Zeiss microscope. The image was captured using a Canon 5D Mark II COMOS sensor coupled with C3C21 colour filter (LOMO) in camera input, to cut off the IR radiation.

3. Results and discussion

3.1. Zirconia nanocrystal up-conversion characteristics

At first, it should be noted that zirconia nanocrystal up-conversion luminescence spectra were environment dependent (figure 1 A). Not only did the luminescence spectral distribution changed, but also the luminescence intensity weakened strongly in aqueous solutions. Therefore we concluded, that large specific surface areas were responsible for strong zirconia nanocrystals interaction with environment.

However, the ZrO_2 nanocrystal up-conversion luminescence did not change if measured in various aqueous media (water, empty YPD broth, ethanol, YPD broth with yeasts). The nanocrystal up-

conversion intensity changed in aqueous media, but the spectral distribution was the same (results not shown).

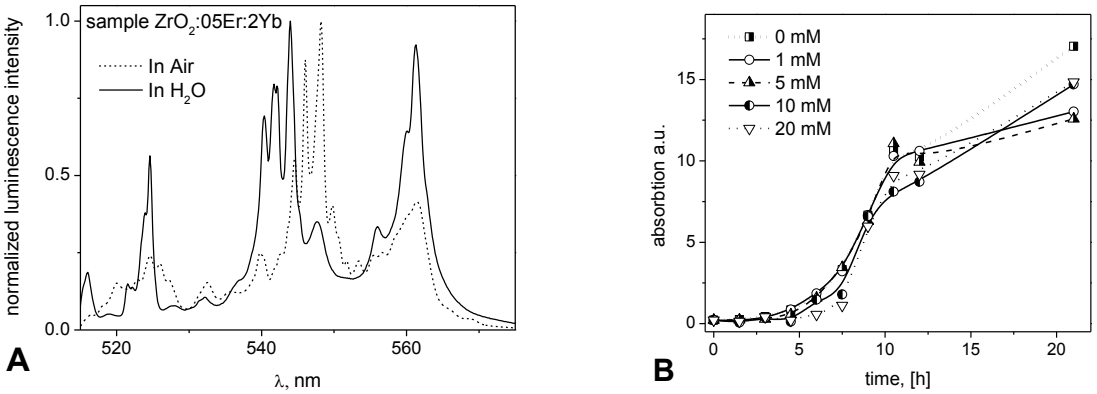

Figure 1. Zirconia nanocrystal up-conversion luminescence (A) and *S. cerevisiae* BY4741 strain growth in broth with different (0-20 mM) concentrations of zirconia nanocrystals (B)

3.2 ZrO_2 nanocrystal toxicity measurements

The toxicity of zirconia nanocrystals on eukaryotic cells was assayed by monitoring baker's yeast growth in YPD broth containing different concentrations (0-20 mM) of zirconia nanoparticles. Similarly to previously reported toxicity assays [16]- OD600 was used to estimate biomass growth in the presence of nanoparticles. Results were plotted as OD600 dynamics over time (figure 1 B). We observed no significant impact of ZrO_2 nanocrystals on *S. cerevisiae* fermentative growth (till 12 hour) over all of the concentrations applied. Similar results in the same concentration range were reported previously with ZnO, TiO_2 and CuO nanoparticles [17]. However, ZrO_2 nanocrystals slowed *S. cerevisiae* growth at concentrations of 1 and 5 mM after glucose exhaustion (after 12 hours of cultivation). Other authors noted that nanoparticles tend to form aggregates in water and microbial cultivation broth; therefore, their toxic effects were not pronounced due to the fact that cells were not exposed to small sized nanoparticles, but rather to their comparatively large aggregates instead [18].

It seemed that ZrO_2 nanocrystals were biologically inert while the cell's respiratory metabolism was suppressed by glucose, and therefore might be used as a biosensor during fermentative growth of yeast. Further investigations should be done to clarify whether rare earth doped zirconia nanocrystals have any specific effects on baker's yeast respiratory growth.

3.2. Up-conversion and TEM imaging

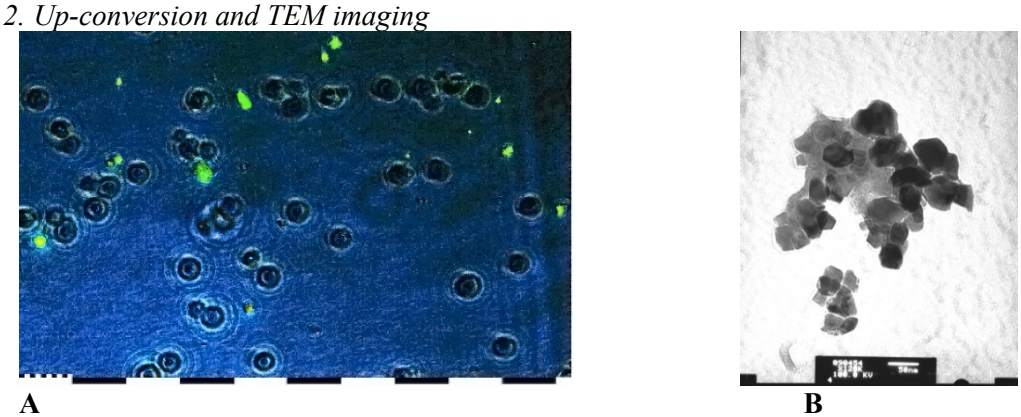

Figure 2. ZrO_2 nanocrystal IR upconversion microscopy (scale bar 10 μm) image with *S. cerevisiae* cells (A) and TEM micrography (scale bar 50 nm) (B)

Zirconia nanocrystals prepared by the Sol Gel method tend to form agglomerates. The IR up-conversion luminescence optical microscope image (Fig. 2 A) showed that there were particles of size under 1 μm, mostly agglomerates; however, it is not possible to determine the agglomerate size at the sub micron level with optical microscopy methods. When the particle size decreases the luminescence intensity decreases too.

The smallest ZrO_2 nanocrystals were possible to observe by TEM (Figure 2 B). Therefore, in order to detect smaller particles by upconversion imaging - the sensitivity of registration system should be increased. Due to low intensity and low resolution we were not able to observe whether uptake of nanocrystals had taken place in yeast cells.

4. Conclusions

The zirconia up-conversion luminescence peak positions were sensitive to the environmental surrounding. However, the presence of yeast cells did not show any significant influence on the spectral composition of the luminescence. Rare earth doped zirconia nanocrystals did not had any toxic effects on yeast cell fermentative growth; besides, it was possible to observe nanocrystals in biological media via upconversion process. Thus, we concluded that it is possible to use zirconia nanocrystals as micro size markers in baker's yeast based bioassays.

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