Analytical Biochemistry xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Notes & Tips

Miniaturized device for agitating a high-density yeast suspension that is suitable for in vivo nuclear magnetic resonance applications

Maksim Zakhartsev [a,*], Christian Bock [b]

[a] Institute for Biochemical Engineering (IBVT), University of Stuttgart, 70569 Stuttgart, Germany
[b] Integrative Ecophysiology, Alfred Wegener Institute for Polar and Marine Research (AWI), 25750 Bremerhaven, Germany

ARTICLE INFO

Article history:
Received 16 August 2009
Available online xxxx

ABSTRACT

In vivo nuclear magnetic resonance (NMR) monitoring requires a high-density cell suspension, where cell precipitation should be avoided. We have designed a miniaturized cell agitator that fits entirely into an 8-mm NMR probe but that, being mounted into the instrument, is situated outside of the sensitive area. The device consists of two glass tubes connected in a way that, when gas flow is blown through them, creates influx of cell suspension into the device that returns through apertures. This flow creates continuous circular vortex of the cell suspension in the whole sample volume, whereas there are no moving mechanical parts or gas bubbles crossing the instrument's sensitive area. The gas flow controls conditions of the cell suspension and removes volatile waste metabolites.

Some methods allow on-line in vivo time-series measurements (i.e., monitoring) of cellular metabolism. For example, nuclear magnetic resonance (NMR)[1] spectroscopy can monitor some important intracellular metabolites, such as phosphorus-containing compounds (e.g., phosphonucleotides, phosphosugars, polyphosphates), in vivo by ^{31}P NMR. With NMR, it is possible to determine in vivo intracellular metabolite concentrations, pH levels, and kinetics of enzyme reactions as well as to identify metabolic pathways (see, e.g., Refs. [1–4]). However, NMR spectroscopy has relatively low sensitivity to physiological concentrations of cellular metabolites. Therefore, limitations of in vivo metabolite concentrations can be overcome only by a high concentration of cells.

Campbell-Burk and Shulman [3] stated that to distinguish molecular species during the course of in vivo NMR measurements, the experimental setup should employ high-resolution spectroscopy, which is possible only if the following criteria are satisfied: (i) high cell density (10–50% wet weight/volume), (ii) a wide-bore NMR instrument (e.g., 20 mm), and (iii) a stirring setup that maintains the constant physiological state of cells within the NMR instrument during the whole measurement period. Narrow-bore ^{31}P NMR spectroscopy (8 mm) also brings the additional advantage of measurements of in vivo kinetics for some reactions using magnetization transfer (see, e.g., Refs. [1,2,4]). However, this method requires long-term accumulation of the signal; therefore, cell precipitation during the course of the measurement must be avoided. Cell precipitation results in heterogeneity of nutrient supply, such as gases and carbon, nitrogen, and phosphorus sources, resulting in variation of the cellular physiological state across the population. Consequently, the cellular precipitation of the suspension must be prevented and, at the same time, all of the required nutrients must be distributed homogeneously to avoid excessive variation of metabolic changes during the NMR monitoring. In addition, the stirring device should not disturb the NMR magnetic field.

A number of approaches have been developed to prevent cell settling during the course of in vivo NMR measurements to achieve a high resolution [3], including a double-bubbler apparatus [5,6], a perfused system by immobilization of cells in an agarose gel matrix [4,7] or other porous materials, a hollow-fiber dialysis system [8], and an NMR bioreactor (e.g., from Bioengineering AG, Switzerland). Of course, an NMR bioreactor directly integrated into the NMR instrument is the best solution for the on-line, in situ, and in vivo measurement of fermentation systems with NMR monitoring of cell cultures growing in a liquid phase (e.g., bacteria, yeast), but this solution is quite expensive.

Yeast *Saccharomyces cerevisiae* strain CEN.PK 122 (from the EUROSCARF yeast collection, http://web.uni-frankfurt.de/fb15/mikro/euroscarf) were grown aerobically in CBS medium [9] in a glucose-limited chemostat at a dilution rate of $D = 0.05\ h^{-1}$ with 18 g L^{-1} glucose in the feeding medium, 30 °C, and 250 rmp. Under these conditions, the biomass density reached 5.36 ± 0.05 gDW (grams dry weight) L^{-1} (or 60.92 ± 2.22 gWW [grams wet weight] L^{-1}). The initial biomass was condensed 40-fold, and 90 ml of yeast culture from the chemostat was pelleted by filtering through a

* Corresponding author. Address: Institute for Biochemical Engineering (IBVT), University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany. Fax: +49 711 685 65164.
E-mail address: maksim.zakhartsev@ibvt.uni-stuttgart.de (M. Zakhartsev).

[1] *Abbreviations used:* NMR, nuclear magnetic resonance; gDW, grams dry weight; gWW, grams wet weight.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.10.011

Please cite this article in press as: M. Zakhartsev, C. Bock, Miniaturized device for agitating a high-density yeast suspension that is suitable for in vivo nuclear magnetic resonance applications, Anal. Biochem. (2009), doi:10.1016/j.ab.2009.10.011

Sartorius cellulose acetate filter (Ø = 0.2 μm) under vacuum and washed three times with working buffer (25 mM Mops [pH 7.0], 2 mM $MgSO_4$, 1.7 mM NaCl, 2 mM KCl, and 100 mM glucose). Then filtered cell pellet was resuspended in 2 ml of the working buffer and 250 μl of D_2O was added (final D_2O content of 11.1%). This was a very high-density cell suspension that would have precipitated within a minute without agitation. After that, 750 μl of this suspension was transferred to an 8-mm NMR probe equipped with an agitating device (Fig. 1).

The cell agitating device was assembled completely from dielectric materials such as glass and silicone tubes, rubber O-rings, and plastic fasteners. The device includes a 200-mm-length glass tube with an external diameter of 4 mm (called the main tube). One of the ends of this tube is stretched out to the capillary with a diameter of 0.1–0.05 mm at the tip. The capillary end of the main tube is inserted into another glass tube with the same external diameter of 4 mm (called the extension tube), and the joint is firmly glued in place (Fig. 1). The length of the extension tube is 60 mm, and it has several 1-mm-diameter apertures close to the glued joint. All apertures must be above the capillary tip (Fig. 1). The main tube is then connected to the gas flow, and the device can be inserted into an 8-mm-diameter NMR tube (8 × 230 mm, Wilmad Labglass, USA). The device is vertically centered within the NMR tube using three 6-mm-diameter rubber O-rings located above the joint between the main and extension tubes (Fig. 1). The device must be immersed in the cell suspension such that the tip of the capillary is under the surface of the cell suspension and the apertures are above the surface of the suspension. When immersed, the main tube of the device sticks out of the NMR tube, and a plastic fastener can be used to secure the depth of the device immersion in the NMR tube. It is important to note that the lower end of the extension tube must be above the sensitive volume of the particular NMR instrument (Fig. 1).

In vivo ^{31}P NMR spectra were acquired at 161.97 MHz on a vertical 9.4T wide-bore NMR spectrometer (Bruker Avance 400 Ultrashield) using an 8-mm 1H/BBI probe with the following parameters: bp pulse, 14 ms (pl 4.6); relaxation delay, 1 s; spectral width, 8090 Hz (corresponding to 50 ppm); time domain, 4K; number of acquisitions, 512 or 1024, with resulting scan time of 11 or 22 min, respectively. Spectra were processed automatically by applying a user's program with size of 16K, line broadening of 5 Hz, and an automatic baseline correction.

Gas (in this case air) was blown through the main tube toward the capillary. The gas passes through the capillary tip and forms a bubble that immediately escapes upward through apertures in the extension tube. The 1-mm gap between the internal wall of the NMR tube and the O-rings of the device is sufficient for the unrestricted gas flow out of the NMR tube. Consequently, it does not cause a buildup of pressure. The movement of gas bubbles pulls the cell suspension into the apertures. After passing the apertures, the gas escapes from the NMR tube, whereas the cell suspension drops back into the tube. The suction force of the agitating device is dependent on the rate of gas flow and is sufficiently strong to result in stirring of the cell suspension across the whole volume of the NMR tube. As a result, there is no cell sedimentation over the measurement period. It is important to note that there are also no gas bubbles crossing the sensitive volume of the NMR instrument (Fig. 1); this is extremely important for the homogeneity of the magnetic field within the sensitive volume of the NMR instrument. In addition, the bottom of the NMR tube was filled with plastic filler (Fig. 1) to reduce tube's internal volume and consequently increase the homogeneity of the suspension.

Furthermore, the gas flow controls experimental conditions (e.g., degree of oxygenation) and also removes volatile metabolites (e.g., CO_2, ethanol) from the cell suspension, whereas nonvolatile

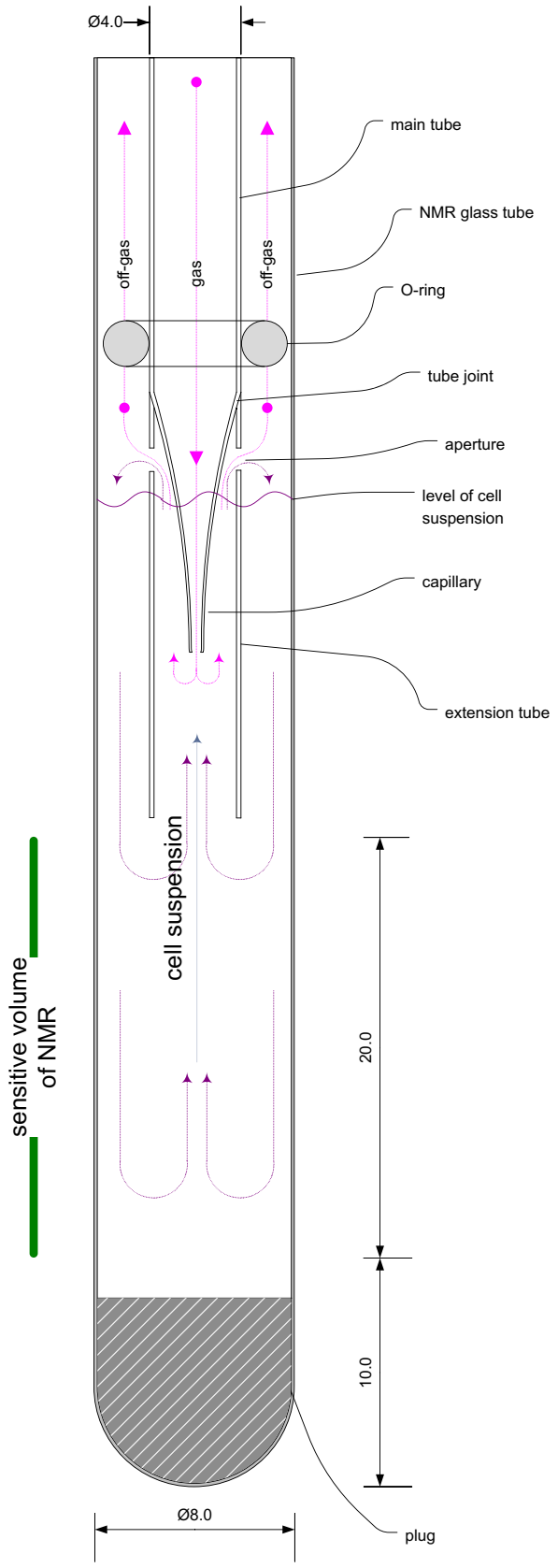

Fig. 1. Sketch of the cell agitating device inserted into a conventional 8-mm NMR glass tube. Blue arrows represent gas flow, and red arrows represent movement of the cell suspension. See the text for further explanations and notation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Please cite this article in press as: M. Zakhartsev, C. Bock, Miniaturized device for agitating a high-density yeast suspension that is suitable for in vivo nuclear magnetic resonance applications, Anal. Biochem. (2009), doi:10.1016/j.ab.2009.10.011

Relative peak areas:

Spectrum	SP	P_i(cyto)	P_i(v)	PP_1	PP_2	PP_n
1	0.51	0.13	1	0.26	0.36	3.66
2	0.67	0.18	1.12	0.32	0.35	3.62

* peak areas are normalized to the P_i(v) value at first scan

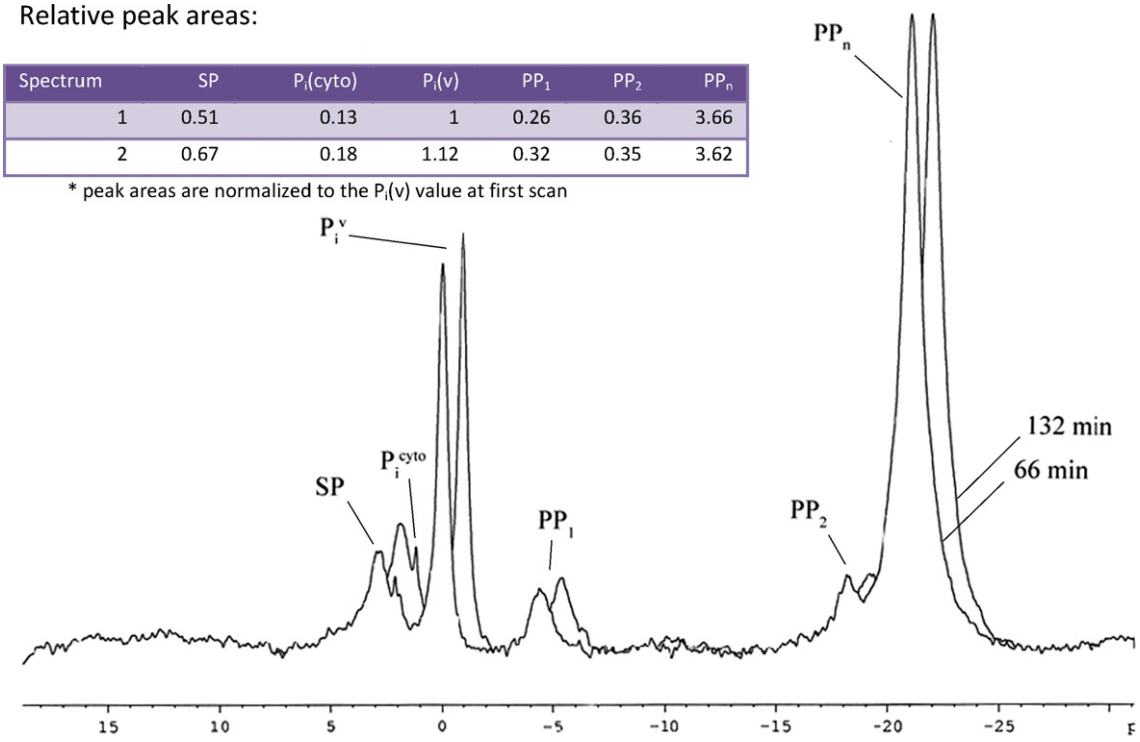

Fig. 2. In vivo ^{31}P NMR monitoring of high-density yeast *S. cerevisiae* CEN.PK 122 suspension agitated using the described device. Shown are two consecutive spectra, each integrated from 3072 scans (66 min). SP, phosphosugars; P_i(cyto), cytoplasmatic inorganic phosphate; P_i(v), vacuolar inorganic phosphate; PP_1 and PP_2, oligophosphates; PP_n, polyphosphates.

metabolic waste products (e.g., glycerol) are either innocuous or nontoxic until they reach high concentrations. In addition, the cell suspension can be fed with concentrated solutions of nutrients through another supply line. Under such conditions, high-density yeast suspensions remain viable and physiologically intact for [illegible]h, which is sufficient time to run high-resolution in vivo NMR measurements in narrow-bore instruments (Fig. 2).

Acknowledgments

The authors thank Rolf Wittig (AWI, Bremerhaven, Germany) for technical support in this research and also thank Glenn Lurman (Institute of Anatomy, University of Bern).

References

[1] K. Brindle, ^{31}P NMR magnetization transfer measurements of flux between inorganic phosphate and adenosine 5′-triphosphate in yeast cells genetically modified to overproduce phosphoglycerate kinase, Biochemistry (Mosc.) 27 (1988) 6187–6196.

[2] S.L. Campbell, K.A. Jones, R.G. Shulman, In vivo ^{31}P nuclear magnetic resonance saturation transfer measurements of phosphate exchange reactions in the yeast *Saccharomyces cerevisiae*, FEBS Lett. 193 (1985) 189–193.

[3] S.L. Campbell-Burk, R.G. Shulman, High-resolution NMR studies of *Saccharomyces cerevisiae*, Annu. Rev. Microbiol. 41 (1987) 595–616.

[4] J.G. Sheldon, S.P. Williams, A.M. Fulton, K. Brindle, ^{31}P NMR magnetization transfer study of the control of ATP turnover in *Saccharomyces cerevisiae*, Proc. Natl. Acad. Sci. USA 93 (1996) 6399–6404.

[5] R.S. Balaban, D.G. Gadian, G.K. Radda, G.G. Wong, An NMR probe for the study of aerobic suspensions of cells and organelles, Anal. Biochem. 116 (1981) 450–455.

[6] J.R. Gillies, J.R. Alger, J.A. den Hollander, R.G. Shulman, Intracellular pH measured by NMR: Methods and results, in: R. Nuccitelli, D.W. Deamer (Eds.), Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions, Alan R. Liss, New York, 1982, pp. 79–104.

[7] K. Brindle, S. Krikler, ^{31}P-NMR saturation transfer measurements of phosphate consumption in *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 847 (1985) 285–292.

[8] G.S. Karczmar, A.P. Koretsky, M.J. Bissell, M.P. Klein, M.W. Weiner, A device for maintaining viable cells at high densities for NMR studies, J. Magn. Reson. 53 (1983) 123–128.

[9] C. Verduyn, E. Postma, W.A. Scheffers, J.P. van Dijken, Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation, Yeast 8 (1992) 501–517.

Please cite this article in press as: M. Zakhartsev, C. Bock, Miniaturized device for agitating a high-density yeast suspension that is suitable for in vivo nuclear magnetic resonance applications, Anal. Biochem. (2009), doi:10.1016/j.ab.2009.10.011