Journal of Bioscience and Bioengineering
VOL. 111 No. 4, 454–458, 2011

www.elsevier.com/locate/jbiosc

Cytotoxicity evaluation of reactive metabolites using rat liver homogenate microsome-encapsulated alginate gel microbeads

Naoko Yamamoto,[1,2] Kikuo Komori,[1,*] Kevin Montagne,[1,3] Hitoshi Matsui,[2] Hidenari Nakayama,[1] Shoji Takeuchi,[1,2] and Yasuyuki Sakai[1,2]

Institute of Industrial Science, University of Tokyo, Komaba, Meguro-ku, Tokyo 153-8505, Japan,[1] Life BEANS Center BEANS Project, Komaba, Meguro-ku, Tokyo 153-8505, Japan,[2] and LIMMS/CNRS-IIS, University of Tokyo, Komaba, Meguro-ku, Tokyo 153-8505, Japan[3]

Received 20 August 2010; accepted 1 December 2010
Available online 15 January 2011

We present an improved cytotoxicity test for reactive metabolites, in which the S9 microsomal fraction of rat liver homogenate is encapsulated in alginate gel microbeads to avoid cytotoxic effects of S9-self-generated toxicants, microsomal lipid peroxides. The S9-encapsulated gel microbeads were prepared by a coaxial two-fluid nozzle and surfaces of the microbeads were coated with poly-L-lysine (PLL). Although the initial metabolic rate of the S9-encapsulated gel microbeads was about 20% slower than that of bare S9, the microbeads prevented the leakage of microsomal lipid peroxides thanks to the dense alginate and PLL polymer networks. In fact, the half maximal effective concentration of the indirect mutagen cyclophosphamide on NIH3T3 cells in the presence of the S9-encapsulated gel microbeads was about 5 times higher than that in the presence of bare S9. Use of the S9-encapsulated gel microbeads enabled the more accurate evaluation of the cytotoxicity of the reactive metabolites without the S9-based cytotoxicity.

[**Key words:** Cytotoxicity test; Rat liver homogenate microsome; Gel microbeads; Encapsulation; Reactive metabolite]

A rapid and simple way to evaluate the toxicity of xenobiotics, such as drugs, pesticides, and food additives, has been increasingly sought for the early stages of their development. During xenobiotic metabolism, a xenobiotic introduced into the human body is often converted into a reactive metabolite, mainly by detoxification enzymes produced in the liver, resulting in expression and/or enhancement of toxicity. Reactive metabolites are widely recognized as mutagens and carcinogens based on their covalent binding with DNA and proteins (1,2). For instance, it is generally known that aflatoxin B_1, which is a natural carcinogen, is itself lowly toxic, while its reactive metabolites, aflatoxin B_1-8,9-epoxide, exhibit severe toxicity to the liver (3,4). Therefore, toxicities of not only xenobiotics but also their metabolites should be determined.

In drug metabolism and detoxification pathways, cytochrome P450 (CYP) enzymes are generally involved in the formation of reactive metabolites and can be isolated in microsomes of human and animal cells. The S9 microsomal fraction of rat liver homogenate is widely used in *in vitro* bioassays to evaluate the toxicity of reactive metabolites (5–7). Since S9 contains enzymes of the CYP superfamily, phase I metabolism in the liver is partially replicable in the presence of cofactors, such as $NADP^+$, which is used as an electron donor. Therefore, comprehensive evaluation of CYP-metabolized xenobiotic toxicity may become possible. However, it is difficult to determine net cytotoxicity of the xenobiotic metabolites, because lipids that make up S9 microsomes are known to be metabolized by CYPs, resulting in the formation of toxic microsomal lipid peroxides (8,9). Therefore, due to the different sensitivity of target cells to S9 and its metabolites, the concentration of S9 is adjusted in cytotoxicity tests in order to minimize cell damage.

To overcome this problem, encapsulation of S9 in hydrogel microbeads may be an effective way of preventing leakage of the microsomal lipid peroxides. It has been reported that hydrogel microbeads are extensively employed as supporting materials for biocatalysts (10), proteins (11,12), and cells (13,14). In particular, there has been considerable research on gel microbeads of alginic acid, which is a well known as polysaccharide, thanks to its possible handling in mild conditions and high biocompatibility (15,16). Moreover, coating the beads with poly-L-lysine (PLL) can control diffusion of chemicals due to the polyion complex-based polymer network (17,18). This means that high-molecular-weight chemicals larger than the polymer network pores can be retained in the gel microbeads, whereas low-molecular-weight chemicals can move in and out of the gel microbeads. Therefore, it is expected that metabolic activity of CYPs in S9 will be retained and leakage of microsomal lipid peroxides prevented by encapsulation of S9 in alginate gel microbeads coated with PLL. Such S9-encapsulated gel microbeads may lead to the formation of artificial liver cells.

In this work, using a coaxial two-fluid nozzle, we fabricated non toxic S9-encapsulated alginate gel microbeads, the surfaces of which were coated with PLL. Moreover, we established a simple *in vitro* cytotoxicity test using an incubation system based on a membrane

* Corresponding author. Tel./fax: +81 3 5452 6349.
E-mail address: kkomori@iis.u-tokyo.ac.jp (K. Komori).

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doi:10.1016/j.jbiosc.2010.12.004

culture insert, on which the S9-encapsulated gel microbeads were seeded. Here, we used a mouse fibroblast NIH3T3 cells as a cell model and the anti-cancer drug cyclophosphamide (CPA) as an example of a reactive metabolite precursor, as CPA is known as an indirect mutagen. Cytotoxicity was determined from the ratio between cell growth after exposure to the xenobiotic and that observed in a standard culture system. The present test might readily permit direct comparison of the cytotoxicity of various S9-generated xenobiotic metabolites under the same concentration of S9, regardless of the type of cell.

MATERIALS AND METHODS

Preparation of gel microbeads Using a coaxial two-fluid nozzle, 2.5 wt.% alginate (Wako Pure Chemical Industries, Ltd., Japan, Mw = 64 000) aqueous solution with 0 or 50% S9 of rat liver homogenate suspension (Kikkoman, Japan), containing 14 μM CYPs, was discharged from the inner nozzle (0.27 mm in internal diameter (ID)) into 5 wt.% $CaCl_2$ aqueous solution stirred at 700 rpm under running N_2 gas (2.0 L min^{-1}) from the outer nozzle (1.3 mm in ID). Microbeads thus obtained were suspended in 1.5 wt.% PLL (Sigma-Aldrich, USA, Mw = 24 000) aqueous solution, followed by suspension in D-MEM/F12 (Invitrogen, USA) containing 10% fetal bovine serum (FBS; Gemini Bio-Products, USA). Consequently, we obtained normal and S9-encapsulated gel microbeads, the surfaces of which were electrostatically coated with PLL and proteins.

Determination of metabolic activity The metabolic activity of CYP 1A1/2 in S9 was determined on the basis of fluorescence intensity of resorufin (λ_{ex} = 530 nm and λ_{em} = 585 nm), which is enzymatically converted from ethoxyresorufin (ER, Sigma-Aldrich) (19–21). 5.0×10^5 S9-encapsulated gel microbeads L^{-1} and 20 μM dicumarol (Sigma-Aldrich) were added to D-MEM/F12-based culture medium containing 10% FBS, 25 mM hydroxyethylpiperadine-N′ 2-ethanesulfonic acid (Dojindo Laboratories, Japan), 100 units mL^{-1} penicillin (Wako), 100 μg mL^{-1} streptomycin (Wako), and 0.25 μg mL^{-1} amphotericin B (Sigma-Aldrich). Then, the culture medium containing S9-encapsulated gel microbeads was gently stirred at 37°C, followed by the addition of culture medium containing 35 μM ER and cofactors, such as 0.83 mM D-glucose-6-phosphate disodium salt hydrate (Sigma-Aldrich), 0.67 mM β-$NADP^+$ (Oriental Yeast Co., Ltd, Japan), and 0.83 mM $MgCl_2$ (final concentrations). A spectrofluorophotometer RF-5300PC (Shimadzu, Japan) was used for spectrofluorometric measurements.

Cytotoxicity tests To evaluate the cytotoxicity of S9-encapsulated gel microbeads, we used a culture insert-based incubation system as illustrated in Fig. 1. A polyester (PE) membrane culture insert (4.67 cm^2 with 0.4 μm pores) filled with S9 or S9-encapsulated gel microbeads in culture mediums was set in the accompanying culture dish, on which NIH3T3 cells were incubated for 24 h after cell seeding at an initial density of 2.0×10^4 cells cm^{-2}. NIH3T3 cells were incubated in the culture medium (4.1 mL in total) containing cofactors for 4 h in a shaking incubator (5% CO_2, 37°C), followed by further incubation in the culture medium without cofactors for 7 days in a stationary incubator (5% CO_2, 37°C) after removal of the culture insert. As a control experiment, NIH3T3 cells were incubated in the culture medium containing S9 and cofactors for 4 h, followed by incubation in the culture medium. Based on 4′,6-diamino-2-phenylindole (DAPI) fluorometry (22), the number of cells on day 7 of cell culture was measured. The number of cells obtained in a standard culture conditions was also measured as a reference.

To evaluate the cytotoxicity of CPA, the measurement system was essentially the same as the one described above. The PE membrane culture insert filled with 4.4×10^2 S9-encapsulated gel microbeads per cm^2 and culture medium was set in the accompanying dish with NIH3T3 cells. NIH3T3 cells were incubated in the culture medium (4.1 mL in total) containing cofactors and CPA for 4 h in the shaking incubator, followed by further incubation in the culture medium without cofactors and CPA for 7 days in the stationary incubator after removal of the culture insert. The number of cells on day 7 of cell culture was measured on the basis of DAPI fluorometry.

RESULTS AND DISCUSSION

Shape of gel microbeads It is known that spherical alginate microbeads are formed when the microbeads are prepared at 1.2 wt.% or higher alginate concentrations (9). The normal and S9-encapsulated gel microbeads prepared for this study and observed under a microscope were spherical (Fig. 2), consistent with the fact that a 2.5 wt.% alginate aqueous solution was used. In addition, the size of normal and S9-encapsulated gel microbeads was found to be 480 ± 20 μm and 720 ± 30 μm in diameter, respectively. This difference may be explained as follows: normal microbeads are densely packed with alginate polymers thanks to coordinate bonds between carboxyl groups via Ca^{2+}. Regarding S9-encapsulated microbeads, since the surface of rat liver microsomes is known to be negatively charged at neutral pH (23), microbeads swelled likely due to electrostatic repulsion between parts of carboxyl groups in the gel microbeads and the surfaces of S9 microsomes. Note that reversible expansion/contraction of a coordination bond-based hydrogel is known to be controllable by electrostatic interaction based on dissociation/formation of the coordination bond (24).

Biochemical characteristics of S9-encapsulated gel microbeads To determine the metabolic capacity of the S9-encapsulated gel microbeads, we measured the activity of CYP 1A1/2 contained in S9 as an example of the CYPs superfamily by measuring the fluorescence intensity of resorufin, the metabolite of ER by CYP1A1/2 activity (19–21). The plot a in Fig. 3 shows that resorufin production in the culture medium containing 5.0×10^5 beads L^{-1} of S9-encapsulated gel microbeads, 35 μM ER, and cofactors increased for up to 2 h and then leveled off. As a control experiment, plot b in Fig. 3 shows resorufin production in the culture medium containing 5% S9, 35 μM ER, and cofactors. Note that the S9 concentration in the culture medium containing 5% S9 was roughly equal to that containing 5.0×10^5 beads L^{-1} of S9-encapsulated gel microbeads. As can be seen in plot a and b of Fig. 3, the final amount of resorufin produced by 5.0×10^5 beads L^{-1} of S9-encapsulated gel microbeads was actually equal to that by 5% S9. However, the initial rate of resorufin production in the former was slower than in the latter. Based on plot a and b in Fig. 3, each initial rate was determined to be 15 and 19 nM min^{-1}, respectively. This difference is likely due to the lower diffusion rates of ER and resorufin in the gel microbeads.

In addition, we examined leakage of S9 from gel microbeads. After S9-encapsulated gel microbeads were removed from the culture medium containing 35 μM ER and cofactors after 30 min, resorufin production was further measured. If resorufin production increased after removal of the S9-encapsulated gel microbeads from the culture medium, S9 might have leaked out of the gel microbeads. However, resorufin production did not increase after 60 min (Fig. 3, plot c). This

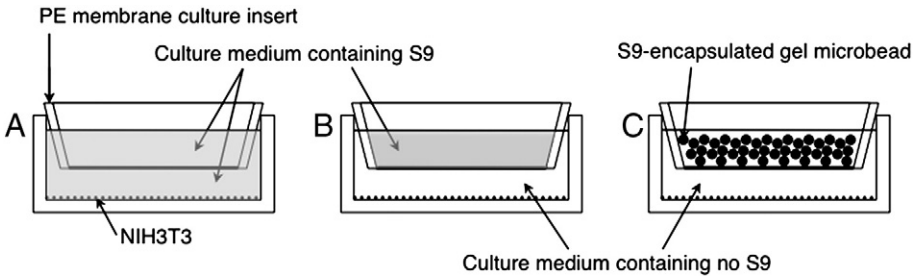

FIG. 1. Schematic illustration of the PE membrane culture insert-based incubation system for the metabolic activation test. (A) S9 is present in both culture insert and culture dish. (B) S9 is present only in the culture insert. (C) S9-encapsulated gel microbeads are present only in the culture insert.