

CHARACTERIZATION OF A SPHEROID FORMED ON THE
SUBSTRATUM CONTAINING A THERMO-RESPONSIVE
POLYMER AND ITS APPLICATION AS AN *IN VITRO* ASSAY
SYSTEM OF *IN VIVO* ACTIVITY OF CELLS

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Objectives

Cells isolated from animal bodies and cultured *in vitro* are very useful and easy to be handled in biological experiments as compared to those *in vivo*. However, it is known that cultured monolayer cells tend to lose both normal functions and morphology. In attempting to obtain an ideal *in vitro* culture system by which we can extrapolate the biological activities of cells in the living body, we have developed a novel culture system of multicellular spheroids called organoids by utilizing a thermo-responsive polymer as a substratum of anchorage dependent cells. The organoid is a three-dimensional mass of cells that mimics an organ of the body. Our final goal is to reconstitute the organoids of various normal organs (e.g. liver, skin, etc.) and also abnormal diseased organs (e.g. tumor). We expect that organoids are very useful in researches such as new drug development, alternatives for experimental animals, artificial organs, etc.

Methods and results

(1) Preparation of a thermo-responsive substratum and recovery of cells cultured on it¹⁾

Poly-N-isopropyl acrylamide (PNIPAAm), a typical thermo-responsive polymer, dissolves in water below a critical temperature and precipitates immediately when the temperature exceeds this critical value.

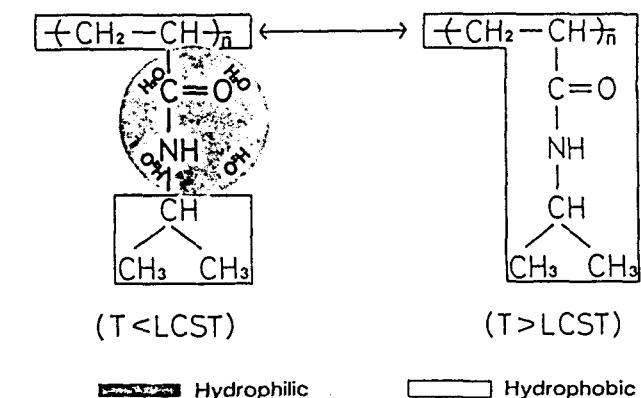


Figure 1. Thermo-responsiveness of PNIPAAm. 1.0 (w/v)% aqueous solution of PNIPAAm prepared below the LCST is insoluble and forms a precipitate at the cell culture temperature of 37 °C, however becomes instantly soluble in water at room temperature. This change from a soluble state to an insoluble state is reversible below and above the LCST of approximately 31.8 °C. The conversion is caused by the temperature-dependent formation and destruction of hydrogen bonds between the water molecules and the amide groups of the PNIPAAm.

The conversion from water-soluble (hydrophilic) and water-insoluble (hydrophobic) states is thermally reversible and the critical temperature is called the Lower Critical Solution Temperature (LCST; about 32 °C) (Fig. 1).

PNIPAAm polymers with weight-average molecular weights of about 2 million were synthesized from N-isopropyl acrylamide (NIPAAm) monomers. We obtained the following results. The LCST of PNIPAAm in water and in culture medium (DMEM supplemented with 10 % heat-inactivated FBS, 20 mM HEPES, 100 units/ml penicillin, and 100 µg/ml streptomycin) are about 31.8 °C and 28.6 °C, respectively. The PNIPAAm polymers have no cytotoxicity although the NIPAAm monomers are cytotoxic. The PNIPAAm possesses no affinity for any kinds of anchorage dependent cells we have checked. Therefore, the thermo-responsive substratum was prepared by pouring the uniformly mixed solution of PNIPAAm and type-I collagen which is a typical adhesive protein onto a culture dish and then drying it aseptically at 10°C (PNIPAAm-collagen substratum; each about 0.1 mg, total about 0.2 mg of the mixture / cm²).

Human dermal fibroblasts suspended in pre-warmed (37°C) culture medium were seeded on the pre-warmed (37°C) PNIPAAm-collagen substratum and cultured at 37°C. The cells attached well, spread, proliferated to the confluent monolayer state, and completely covered the substratum. By simply decreasing the ambient temperature from 37°C to a temperature below the LCST, the cells were completely detached from the substratum as a cell sheet without the use of conventional detaching agents such as trypsin and EDTA.

(2) Preparation of the multicellular homo-spheroid and its histological and biochemical characterization^{1, 2)}

The confluent human dermal fibroblasts on the PNIPAAm-collagen substratum were forced to detach as a cell sheet by decreasing the ambient temperature below the LCST. The detached cell sheet was cultured at 37°C on the non-adhesive substratum of PNIPAAm (PNIPAAm substratum; about 0.1 mg / cm²). The floating monolayer cell sheet, which is made of polygonal cells linked together with many microvilli, shrank and condensed into an aggregate with gap and tight junctions within several hours and finally formed a multicellular spheroid by 2 days (Fig. 2).

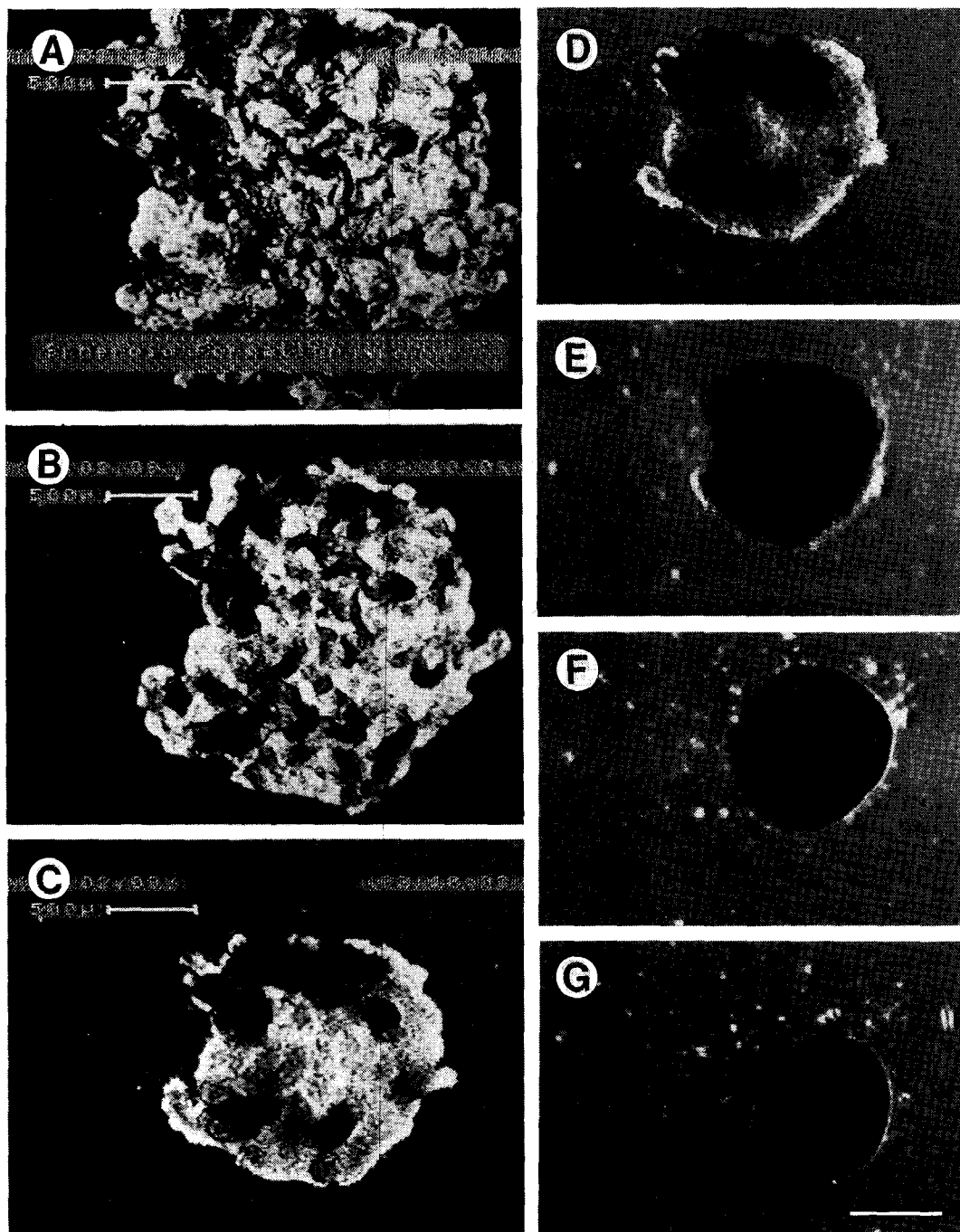


Figure 2. Phase-contrast microphotographs of multicellular spheroid formation. A cell sheet was detached from the PNIPAAm-collagen substratum with a surface area of 1.1 cm^2 . (A) Immediately after complete detachment, (B) 1 h in culture, (C) 3 h, (D) 6 h, (E) 12 h, (F) 24 h, (G) 48 h. (Bar= $500 \mu\text{m}$)

Spheroids were covered with squamous cells and contained cuboidal cells inside. Cycloheximide and actinomycin D reversibly inhibited the spheroid formation. Extracellular matrix fibrils deposited among inner cells when the culture period extended over 7 days. A 28-day-old spheroid with a diameter of about 600 μm contained live cells even in the central region. Cellular metabolic activities such as glucose consumption, lactic acid production, and ATP content of spheroids were significantly lower than those of monolayers.

(3) Preparation of the multicellular hetero-spheroid by the over-lay seeding and its histological and biochemical characterization³⁾

We first cultured human dermal fibroblasts (mesenchymal cells) on the PNIPAAm-collagen substratum up to a confluent state, and then seeded rat primary hepatocytes, human epidermal keratinocytes, or human cholangiocarcinoma cells (epithelial cells) onto the fibroblast monolayer. The hetero-spheroid was prepared by detaching the epithelial cell-attached mesenchymal cell monolayer at a temperature below the LCST and culturing it on the non-adhesive PNIPAAm substratum (Fig. 3). The surface area of the substratum and the seeding population ratio of each cell precisely and reproducibly regulated the size and the cell composition of the resulting hetero-spheroid. The hetero-spheroids were immunohistochemically investigated by using antibodies against rat albumin, carboxylesterases, keratin, and CA 19-9, depending on the type of epithelial cells of spheroids. In the hetero-spheroid composed of fibroblasts and hepatocytes, both types of cells were randomly distributed in the spheroid which was fully covered with a few layers of squamous fibroblasts and the hepatocytes expressed albumin and carboxylesterases for a long time.³⁾ Also, the hetero-spheroid was well vascularized by host blood capillaries when it was implanted into nude mice.⁴⁾ In the hetero-spheroid composed of fibroblasts and keratinocytes, the keratinocytes migrated to the surface and expressed differentiated characters.⁵⁾ And in the hetero-spheroid composed of fibroblasts and cholangiocarcinoma cells,

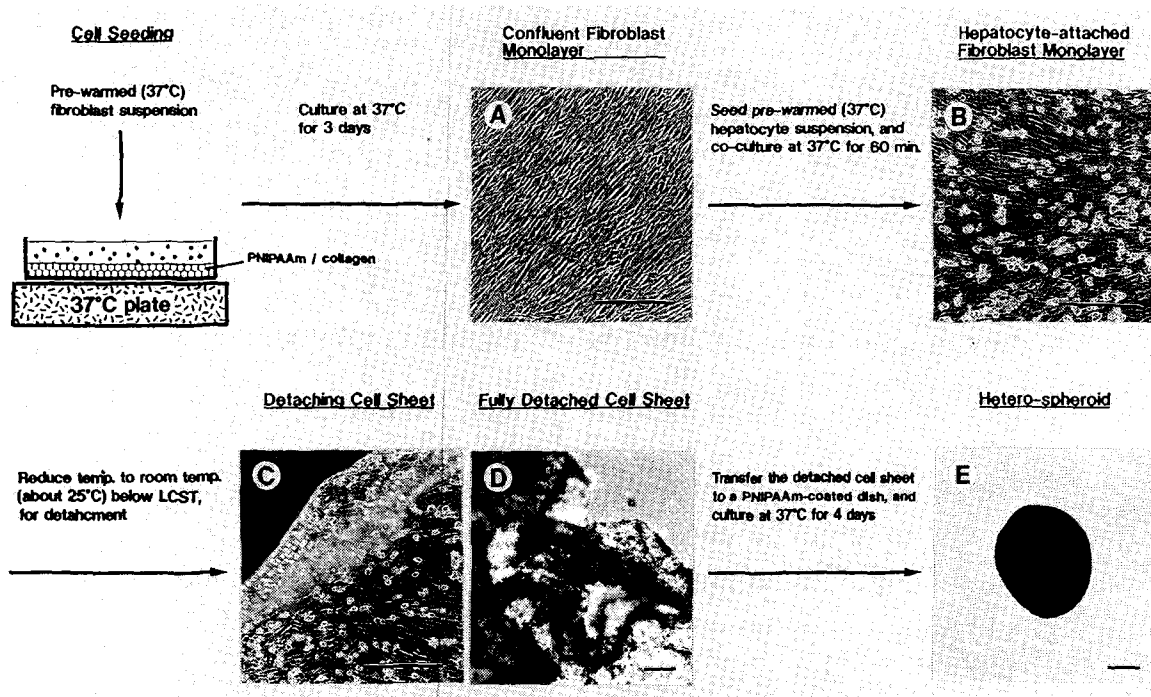


Figure 3. A scheme illustration of the preparative processes of a hetero-spheroid composed of human dermal fibroblasts and rat hepatocytes. (Bar=300 μ m)

the carcinoma cells formed duct structures which are similar to the original cancer tissue *in vivo*.⁶⁾

(4) Preparation of the multicellular hetero-spheroid by the mixture seeding and its histological and biochemical characterization⁷⁾

The mixture of rat liver parenchymal and non-parenchymal cells were seeded on the PNIPAAm-collagen substratum and cultured up to a confluent state. The hetero-spheroid was prepared by detaching the monolayer at a temperature below the LCST and culturing it on the non-adhesive PNIPAAm substratum. The hetero-spheroid composed of rat liver parenchymal and non-parenchymal cells had an ability to secrete albumin and showed hydrolase activity towards p-nitrophenyl acetate (PNPA) over a period of 60 days (Fig. 4).

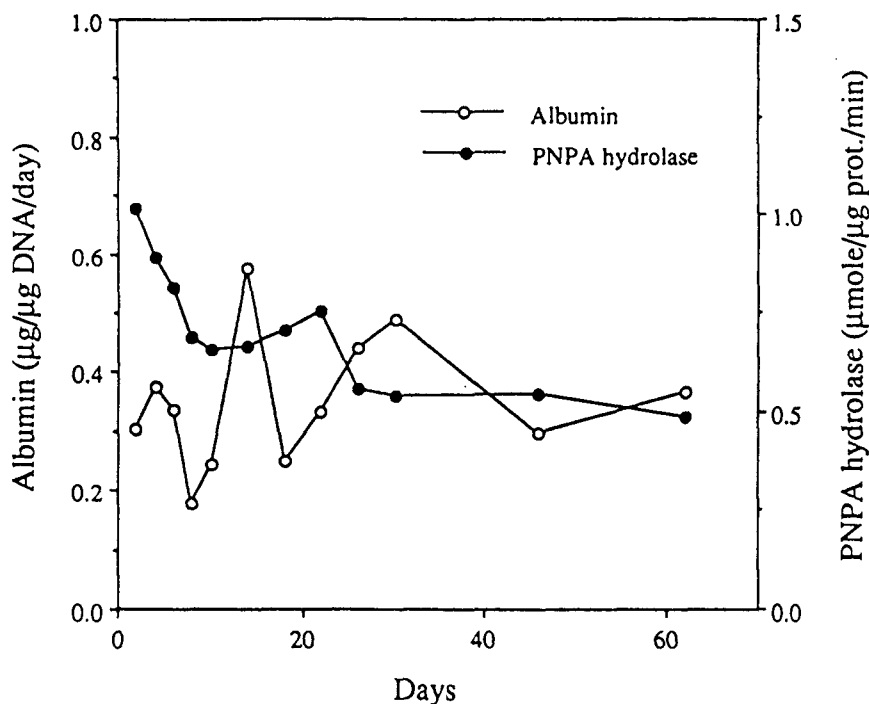


Figure 4. Time course of albumin secretion in the medium and PNPA (p-nitrophenyl acetate) hydrolase activity of cultured spheroids. The content of DNA and protein, and PNPA hydrolase activity was determined for homogenates of three spheroids.

Conclusions

These findings suggest that the method described above is useful for making a hetero-spheroid that morphologically and functionally resembles tissues or organs *in vivo*. We conclude that the hetero-spheroid developed in the present study can be called the organoid and utilized as an *in vitro* model of living organs for studying characters of cells in a condition that resembles the *in vivo* state. This culture system will be useful to approximate the *in vivo* state much more closely than the conventional two-dimensional cell culture system.

Acknowledgments

We thank our collaborators Dr. Yuichi Mori of Japan Research Center, W. R. Grace & Co.-Conn. for the thermo-responsive polymer work, Dr. Koichi Ueno of Pharmaceutical Sciences, Chiba University for the hepatocyte spheroid work, Drs. Yo Mori and Akira Ito of Department of Biochemistry, Tokyo College of Pharmacy for the skin cell spheroid work, Dr. Sunao Kubota of Department of Surgery, St. Marianna University School of Medicine for the cancer cell spheroid work, and Dr. Tomohide Yonaha of Electron Microscope Laboratory, St. Marianna University School of Medicine for the electron microscopic observation of spheroids.

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