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Adhesive cell cultivation on polymer particle having grafted epoxy polymer chain

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ABSTRACT

In this study, we synthesized a new cell immobilization support having poly(glycidyl methacrylate) as a graft polymer chain and used this support for cell cultivation. Base polymer particle was synthesized by suspension polymerization and epoxy polymer chain was extended from particle surface on graft polymerization. Produced polymer particles had broad particle size distribution ranging from 20 to 1000 μm and the degree of polymerization of grafted polymer chain was ranged from 500 to 1000. The effects of various factors, such as grafted polymer chain length and its surface density, composition of base polymer network and graft polymer chain, on the cell growth of murine fibroblast cell line (MS-5 cell) on polymer particle were studied. This polymer particle could cultivate not only fibroblast cell line but also epidermal cell line (HeLa cell), osteoblast cell line (MC3T3E1 cell), and chondrocyte cell line (ch-8 cell) on its surface. Growth rate is almost the same as that of cells using poly(styrene) tissue culture dish. To apply this cell cultivation system for examination of cell co-culture, HeLa cell immobilized on 100 μm of polymer particle was successfully co-cultured with MS-5 cell immobilized on 300 μm of polymer particle for four weeks.

1. Introduction

Cell-to-cell adhesions and cell-to-cell junctions play various important roles, such as forming and maintaining of tissues and organs, protection against bacteria penetration, and support for cell differentiation (Hubbell, 1995; Sternlicht and Werb, 2001; Alberts et al., 2002). Various proteins required for cell adhesion and cell junction have been identified and their roles have been clarified from many studies concerning for molecular biology. However, the mechanisms of signal transduction and cell differentiation induced by cell adhesion and cell junction *in vivo* have been poorly understood. This was because organs and tissues were heterogeneous and signal transduction between cells was composed of many kinds of molecules. Therefore, it was very difficult to identify and isolate activated cell site and to evaluate its functions correctly. Furthermore, adhesive cell cultivated by ordinary dish culture spreads two-dimensionally and could not form three-dimensionally structure, such as organs and tissues. Many researchers have desired to reconstruct organs and tissues *in vitro* (Soria et al., 2000; Poumay and Coquette, 2007; Neuss et al., 2008).

Recently, using cell sheet, new tissue reconstruction technology have been developed and applied for regenerative medicine (Elloumi-Hannachi et al., 2010). In this technology, cell was cultivated on thin thermo responsive polymer sheet mainly composing of poly(*N*-isopropylacrylamide) (PIPAAm) and thermal phase transition successfully divide cell sheet from base thin polymer film (Shimizu et al., 2001, 2003; Sasagawa et al., 2010). When adhesive cell was cultivated in dish, suspension of cell was prepared by using trypsin to divide cell from dish surface. In this situation, cell to dish conjugation was enzymatically digested and cell to cell conjugation was simultaneously digested. However, cell sheet prepared by cell sheet technology had not only extracellular matrix between cell and adhered base polymer sheet but also cell-to-cell junctions between cells (Kushida et al., 1999). Using cell sheet technology, the regeneration of myocardium, cornea, skin, periodontium, blood vessel, and so on, have been applied for clinical applications (Elloumi-Hannachi et al., 2010) and good clinical results were obtained for long term tissue preservation (Ide et al., 2006).

Cell sheet was good technology for reconstruction of homogeneous tissue. However, to build complex organization composed of heterogeneous cells, such as hematopoietic microenvironment in bone marrow and pancreas tissue for product of insulin, three-dimensional cell support and growth system in which heterogeneous cells were preserved without growth rate selection

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must be developed. For large scale cultivation of adhesive cell in fluidized bed, fixed bed, and rotating wall vessel reactor, polymer particle (microcarrier) whose size was micron order was effective and they can immobilize various cells having various growth rates (Bryan, 2000; Pörtner et al., 2005). Many researchers have studied the effects of microcarrier surface properties such as chemical nature, charge density, roughness, wettability, rigidity and so on, on cell attachment and growth (Kato et al., 2003 and references therein). Among these properties, the amino group content has been considered a critical factor for cell attachment and growth (Kato et al., 2003). A kind of short linear oligopeptides (Arg-Gly-Asp, RGD sequence) had an important function for cell adhesion (Mizuno and Glowacki, 1996; Wang et al., 2002; Reeve et al., 2003; Zheng et al., 2004; Kurihara and Nagamune, 2005; Bigi et al., 2005). Coating of extracellular matrix, such as collagen, laminin, and fibronectin, was effective to cultivate adhesive cell on artificial material. However, suitable extracellular matrix required was dependent on the difference in the cell species. If extracellular matrix required for cell growth and cell immobilization and secreted from cell is *in situ* immobilized on a support, we can establish new cell immobilization system. This was the starting point of our research.

In our previous study, we developed new graft-polymerization method using a polymerizable azo initiator, 2,2′-azobis[*N*-(2-propenyl)-2-methylpropionamide] (APMPA) having both two vinyl groups and one azo group (Yasuda et al., 2010a). This method can be applied for suspension polymerization and we can easily synthesize polymer particle having grafted functional polymer chain. When glycidyl methacrylate was used for graft co-polymerization, epoxy grafted polymer chain which can rapidly react with amino group of protein was introduced into particle surface. We assumed that a thin layer formed by the reaction between proteins orientated from cell secretion or dissolving in serum and epoxy group of grafted polymer chain enhanced cell adhesion and growth on both particle surface and particle gap. Furthermore, oxygen and nourishment transport through this thin layer would increase the number of cell grown on both particle surface and particle gap and enable long term cultivation.

Therefore, the objective of this work was development of new three-dimensional cell cultivation method using this graft polymer particle composed of poly(glycidyl methacrylate) and optimization of various factors for cell adhesion and growth. For this purpose, the effects of graft polymer chain length, its surface density, composition of base polymer network and graft polymer chain on the cell growth of murine fibroblast cell line (MS-5 cell) on polymer particle were firstly studied. Then, to apply this cultivation method for other kind of adhesive cell and co-culture, epidermal cell line and osteoblast cell line was cultivated in the presence of grafted polymer particle. MS-5 cell cultivated for 5 days using grafted polymer particle was added to HeLa cell whose doubling time was 5-fold faster than that of MS-5 cell and cultivated for long term.

2. Materials and methods

2.1. Materials

2,2′-Azobis(isobutyronitrile) (AIBN), 2,2′-azobis[*N*-(2-propenyl)-2-methylpropionamide] (APMPA), 1,4-butanediol diglycidyl ether, 1,4-dioxane, 2-(diethylamino)ethyl methacrylate (DEAEMA), diethylaminoethyl methacrylate, ethanol, glycidyl methacrylate (GMA), hydrochloric acid, 2-hydroxyethyl methacrylate (HMA), methacrylic acid (MA), methanol, methyl methacrylate (MMA), pentaerythritol triacrylate (PETA) and trypan blue were purchased from Wako Pure Chemical Co. (Osaka, Japan). Cresol red, hydroquinone, phosphoric acid, poly(vinyl pyrrolidone) K-90 (molecular mass is about 360,000) (PVP), sodium hydroxide, toluene were purchased from Nacalai Tesque (Kyoto, Japan). Coomassie brilliant blue G-250, and pentaerythritol triacrylate were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Bovine serum albumin (BSA) (molecular mass is about 66,000) was purchased from Invitrogen (Carlsbad, CA, USA). All of the reagents were used without purification.

2.2. Preparation of polymer particle having graft polymer chain

We used polymer particle having graft polymer chain as cell support. In 1000 ml of three-neck flask, 2.0 g of AIBN, 3.0 g of APMPA, 38.0 g of MMA, 38.0 g of PETA, and 560 ml of water containing 1 wt% PVP were added. The reaction mixture stirred at 70 °C with 300 rpm for 3 h and then at 80 °C with 300 rpm for 2 h. Produced polymer particle having azo group derived from APMPA was washed with distilled water and methanol for three times by repeating suspension and filtration of the particle. Next, in 1000 ml three-neck flask, 10 g of polymer particle having azo group, 0–25 g of GMA, 0–25 g of comonomer, and 250 g of toluene were added. The reaction mixture was stirred at 100 °C with 150 rpm for 8 h (standard condition). Produced particle having grafted polymer chain was washed as described above. Here after, we call polymer particle having graft polymer chain as epoxy grafted polymer particle.

For the preparation of poly(propylene) (PP) plate having graft epoxy chain, 10 mm × 50 mm of PP plate was put on glass laboratory dish and was irradiated with 125 kGy (25 kGy h^{-1} × 5 h)of γ-ray. In N_2 atmosphere, PP plate, 5 g of MA, 20 g of GMA, 8 g of sodium dodecyl sulfate, and 400 g of Milli-Q water was added to a 100 ml of flask and the reaction mixture was incubated at 60 °C for 8 h. After reaction, plate was rinsed with methanol and water.

Polymer particle and PP plate was dipped in ethanol and stored at 4 °C. Prior to particle addition to cell, ethanol was removed by decantation or centrifugation. Polymer particle and PP plate were washed in several changes of Milli-Q water.

2.3. Analysis of polymerization and produced particle

Monomer conversions of polymerization were measured by gravimetric method (Yasuda et al., 2001). Each particle diameter distribution was measured using a laser particle diameter analyzer MICROTRAC FRA (Microtrac Inc., Montgomery, PA, USA). Amount of epoxy group of polymer particle was measured by hydrochloric acid-dioxane method (Weiss and Frederic, 1970). Particle surface was analyzed as tapping method using an atomic force microscope (SPM-9500J3; Shimadzu, Kyoto, Japan). Adsorb rate of BSA to polymer particle was measured by Brad-ford method (Bradford, 1976). BSA size in buffer solution was measured using a Zetasizer Nano ZS (Malvern). The particle pore and its distribution were measured by mercury porosimetry (Pascal 140 and 240, Thermo Scientific, Waltham, MA, USA). About 0.5 g of sample was charged to the glass sample electrode tube (dilatometer CD-3PL, Thermo Scientific) and degassed by Pascal 140 for 30–60 min and mercury was introduced into the pore. Pascal 140 can measure micrometer level of pore (>3 μm). Macro-pore and meso-pore ranged from 3 μm to 7 nm was measured by Pascal 240 after the measurement of Pascal 140 in which particle large gap was filled with mercury. The dilatometer filled with mercury using Pascal 140 was placed in the oil cell and air was eliminated by filling oil. Pascal 240 added pressure on glass sample electrode tube to fill mercury against meso-pore until 200 MPa. Both results were combined by programs installed by control PC. To check epoxy group introduced into PP plate, IR spectra of PP plate was measured by FT-IR spectrophotometer (FT-IR-410, JASCO, Tokyo, Japan) attached with ATR measurement unit (ZnSe prism).