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SYNTHETIC HYDROGEL MICROSPHERES AS SUBSTRATA FOR CELL ADHESION AND GROWTH

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SUMMARY

Cross-linked poly(methyl methacrylate) (PMMA) microspheres were subjected to alkaline hydrolysis to obtain hydrophilic microspheres having carboxyl residues distributed throughout the matrix. These microspheres were found to support the growth of human skin fibroblasts and human heart and lung cells. Further, fibroblasts grown on them were found to be comparable with those grown on the commercial tissue culture plate with respect to [^{14}C]amino acid uptake and incorporation into proteins. The hydrolyzed PMMA microspheres may find application as a microcarrier for cell culture.

Key words: hydrogel microspheres; cell culture; microcarrier culture.

INTRODUCTION

Mammalian cells normally adhere to a wide range of nonbiological substrates such as polystyrene, porous silica, polyacrylamide, and sephadex (1,10,20,22). Synthetic hydrogels, mostly based on poly(2-hydroxyethyl methacrylate) (poly HEMA) have been studied as possible culture surfaces (5,7,8,14). In a recent report (15) it was shown that surface hydrolysis of poly HEMA using concentrated sulphuric acid can generate a suitable culture surface for the attachment and growth of vascular endothelial cells. It was demonstrated that the methacrylic acid groups produced by hydrolytic etching on the poly HEMA surface were responsible for cell adhesion and growth.

In this study we have attempted to hydrolyze poly(methyl methacrylate) (PMMA) microspheres cross-linked with ethyleneglycol dimethacrylate (EGDM), using concentrated alkali in ethylene glycol (EG) to generate methacrylic acid groups throughout the matrix and to demonstrate that the hydrolyzed microspheres support the growth of human skin fibroblasts and human heart and lung cells. Unlike HEMA, the methyl methacrylate monomer could be easily polymerized in suspension to obtain smooth and perfectly spherical microspheres of desired size. We have recently shown that alkaline hydrolysis of cross-linked PMMA microspheres can generate smooth, perfectly spherical, highly transparent beads having the desired degree of swelling in water (6). These microspheres were found to be supportive of cell growth. With the exception of polyacrylamide hydrogel, to the best of our knowledge, there has been no report on the use of synthetic hydrogels for microcarrier culture.

MATERIALS AND METHODS

Lyophilized tissue culture medium, fetal bovine serum, and trypsin were obtained from GIBCO Laboratories, Grand Island, NY. Penicillin, gentamicin, collagenase, and other fine chemicals were obtained from Sigma Chemical Company, St. Louis, MO. [^{14}C]Amino acid mixture (*Chlorella* protein hydrolysate, sp. activity 20 mCi/m atom C) was purchased from Bhabha Atomic Research Centre, India. Commercial tissue culture plates (surface area, 21 cm^2) were obtained from Nunc (Kamstrup, Denmark).

Dulbecco's modified Eagles medium (DMEM) was prepared from the commercially available powder supplemented with 3.7 g $NaHCO_3$/liter. Lysis buffer consisted of 50 m*M* Tris-HCl (pH 7.4), 0.1% Triton X-100, 0.02% sodium dodecyl sulphate, 150 m*M* NaCl, and 5 m*M* EDTA.

Preparation of the Hydrogel Microspheres

The hydrogel microspheres employed in this study were derived from PMMA beads cross-linked with 1 mol% EGDM by alkaline hydrolysis in EG at 180 to 183° C. Details of the method of preparation of PMMA microspheres, the hydrolytic procedure, and their properties will be reported elsewhere, but briefly the procedure was as follows. Distilled inhibitor-free MMA (BDH, Poole, England) was suspension polymerized in aqueous medium using benzoyl peroxide as the initiator in the presence of 1 mol% EGDM as the cross-linking agent. A combination of polyacrylamide and poly(vinyl alcohol) served as the suspension stabilizer. After polymerization, the beads formed were filtered, washed several times with water, and dried. Beads of different size ranges were then separated by passing them through test sieves.

The microspheres were then hydrolyzed by the following procedure. A 100-ml, round-bottomed flask, fitted with a reflux condenser and a thermometer, was charged with 50 ml of EG containing 5 wt% NaOH. The contents were heated in an oil bath kept over a magnetic stirrer, and the mixture was stirred using a Teflon-coated magnetic stirring bar. Once the solution reached 180° C, 1 g of PMMA beads (355 to 425 μm) was introduced into the flask and the heating continued. The reaction was quenched at the end of 90 min by pouring the contents into a large volume of water. The hydrolyzed beads were filtered, washed, and equilibrated in distilled water for several days with several changes of water.

The hydrolyzed beads showed an equilibrium water uptake of 94 ± 1%, determined from the weight of the swollen and of the dry beads. The swollen beads were found to have a specific gravity of 1.027 in 0.9% NaCl solution (determined using n-heptane as the liquid by the volume displacement technique). The carboxyl groups generated by hydrolysis on the beads were distributed throughout the matrix of the polymer as evidenced by the fact that the beads became completely dyed in methylene blue solutions. Examination of 5-μm thick sections of the methylene blue-dyed microspheres by optical microscopy revealed that the dye was bound uniformly throughout the bead. The amount of carboxyl groups in the microspheres was estimated by acidification and back titration against standard base. The carboxyl content was found to be 43.5% and was reproducible within ± 1%.

Cell culture. Human fetuses were obtained after medical termination of pregnancy in the early weeks of gestation (8 to 10 wk). Primary cultures of human skin fibroblasts and human heart and lung cells were maintained in DMEM containing 20% fetal bovine serum, penicillin (1 mg/ml), and gentamicin (0.05 mg/ml). The microspheres (total surface area, 20 cm^2) were placed on the tissue culture plate, and cells were seeded at a density of about 5 × 10^3 cells/cm^2. Commercial tissue culture plates were seeded in an identical manner for comparison. Growth was monitored in terms of cell count using a hemacytometer and cellular protein content by Lowry's method (13). For cell count, the microspheres were removed from the tissue culture plate and the cells on them and the plate were released using a 1:1 mixture of 0.025% trypsin in Ca-, Mg-free phosphate buffered saline and 0.02% EDTA in Ca-, Mg-free phosphate buffered saline. For protein estimation, the microspheres bearing the cells were washed with phosphate buffered saline and the cells on the microspheres and the plate were digested with 0.1 *N* NaOH. Protein adsorbed on the microspheres was subtracted. Cells grown on the microspheres were visualized by inverted phase contrast (Nikon) and scanning electron (Cambridge) microscopy.

Scanning electron microscopy. Cells on the microspheres were fixed with 2% phosphate buffered glutaraldehyde for 2 h and postfixed with 1% osmium tetroxide for 1 h. After rinsing in phosphate buffer, dehydration was carried out using graded series of alcohol. The microspheres with the cells were vacuum dried after transferring from 100% alcohol through liquid N_2. They were mounted on stubs smeared with sticky conducting paint, coated with silver in a vacuum

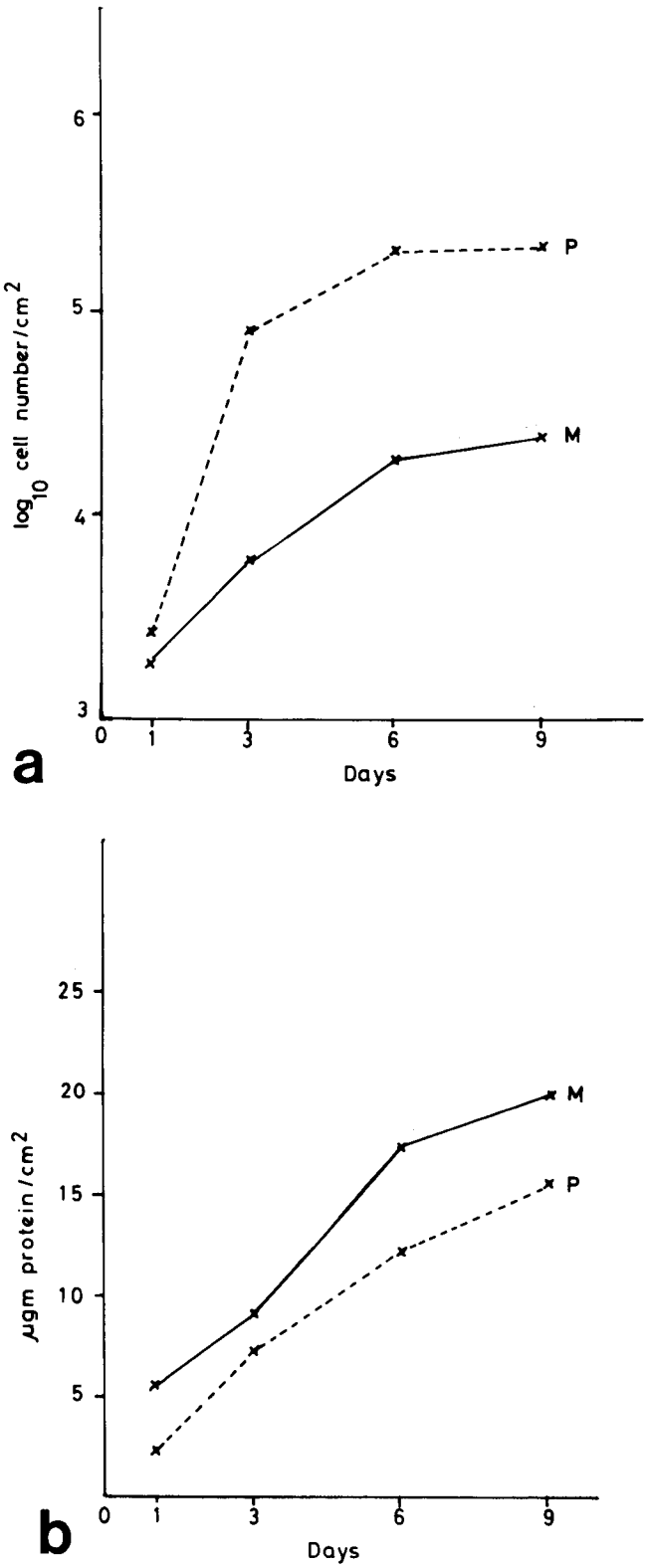

FIG. 1. Growth of human skin fibroblasts on hydrogel microspheres (*M*) and the commerical tissue culture plate (*P*). *a*, In terms of cell count; *b*, in terms of cellular protein content.

coating unit, and examined by scanning electron microscopy.

[^{14}C]*Amino acid uptake and incorporation.* Cells from 3-d-old cultures were washed 3 times with Hanks' balanced salt solution (HBSS) and incubated for 30 min in 3 ml of HBSS containing 3 μCi of the [^{14}C]amino acid mixture. At the end of the incubation, the cells were washed 3 times with HBSS and lysed with the lysis buffer. Cell debris was removed by centrifugation at low speed. One aliquot was used for protein estimation by Lowry's method. A second aliquot was spotted on Whatman no. 3 filter disc, dried, and radioactivity was determined using a liquid scintillation spectrometer (LKB, Sweden). A third aliquot was precipitated with 10% ice-cold trichloroacetic acid and treated as described earlier (19). The final pellet was dissolved in a minimal volume of 0.1 *N* NaOH, spotted on Whatman no. 3 filter disc, dried, and radioactivity was determined.

Results

Growth of fibroblasts on the hydrogel, as monitored in terms of cell number and cellular protein content (Fig. 1 *a*, *b*), indicated that the surface is growth-supportive. Intriguingly, although cell count indicated higher values for the tissue culture plate as compared to the hydrogel, protein content indicated the contrary. The possibility that cells grown on the hydrogel surface have a higher protein content was ruled out by the observation that the same number of cells from the two surfaces had the same protein content as determined by Lowry's method (data not given).

Phase contrast microscopy (Fig. 2 *a*, *b*) and scanning electron microscopy (Fig. 3 *a*, *b*) of fibroblasts and human lung (predominantly epithelial) and heart predominantly myocardial) cells clearly showed that these different cell types adhere well to the hydrogel surface. Fig. 3 *a*, *b* are scanning electron micrographs of multiple layers of human lung cells around the beads. Fig. 3 *b* shows filopodia extending from the cell to the substratum.

Having demonstrated the suitability of the material as a surface for cell adhesion and growth, we wanted to determine whether the cells grown on it show any significant alteration in cellular metabolism. For this, fibroblasts grown on the hydrogel surface were compared with those grown on a commercial tissue culture plate with respect to the uptake of [^{14}C]amino acids and their incorporation into proteins. As is evident from Table 1, it was found that the cells grown on the two surfaces are comparable with respect to the two parameters examined.

Discussion

The microspheres used in this study have been found to support the growth of anchorage-dependent human skin fibroblasts and human heart and lung cells (Figs. 1-3). Further, the results presented in Table 1 show that the material used in this study provides a surface suitable for growth without alteration in at least the two parameters that we have studied. The surface supports cell adhesion by itself without the need for derivatization with adhesion promoting glycoproteins such as fibronectin. It is reasonable to presume that incorporation of adhesion-promoting glycoproteins into the surface would further improve the yield of cells. A comparison of the yields from the two surfaces (Fig. 1 *a*, *b*), as determined by cell count and cellular protein content, presents a discrepancy which we are unable to explain in the light of available data. Direct counting of enzymatically harvested cells can be misleading, because although relatively sparse cultures can be readily dispersed into single cell suspensions, it can be difficult to obtain, enzymatically, such suspensions without damaging some cells from dense cultures (12). It is likely that trypsin, at the concentration employed, is unable to detach all the cells from the spherical surface, yielding an apparently lower cell count. Higher concentrations of trypsin were not

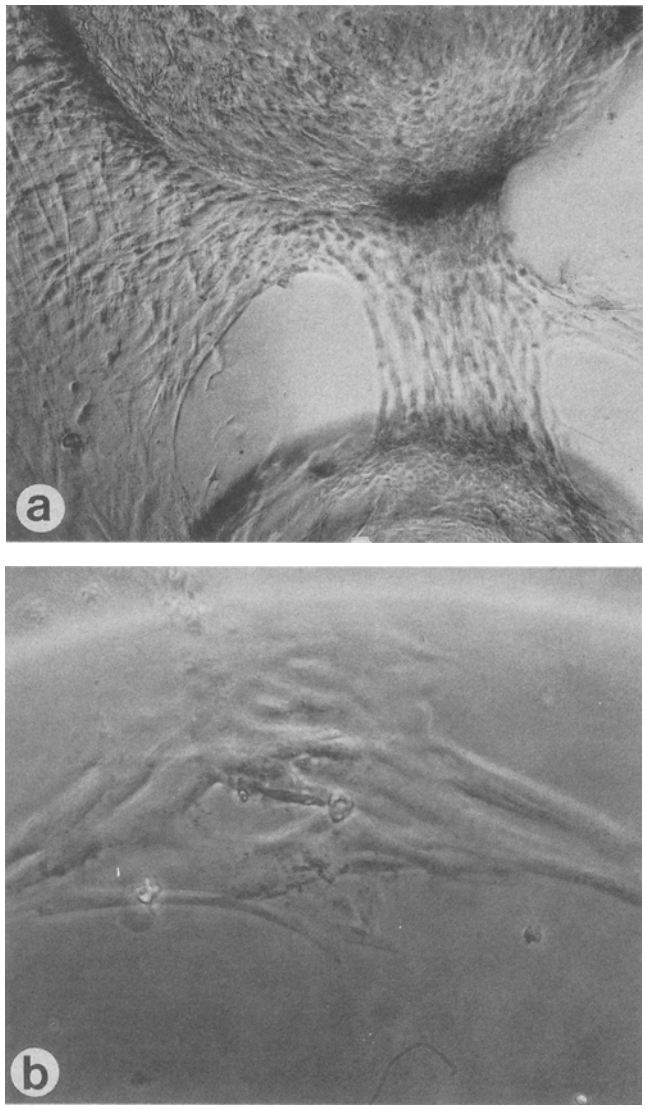

FIG. 2. Phase contrast micrographs. *a*, Human skin fibroblasts. ×142. *b*, A group of human heart cells grown on the hydrogel microspheres. ×285.

used, to avoid damage to the cells. On the contrary, digestion of the cells on the microspheres using 0.1 *N* NaOH would ensure total extraction of cellular protein from the material. There could also be differences in the amount of extracellular matrix elaborated by cells under different culture conditions (12).

It is pertinent to point out that the material offers many advantages as a surface for cell culture. The small spheres provide a higher surface area-to-volume ratio which would ensure higher total yield of cells for a given volume compared to flat surfaces. Although we have done static cultures, microspheres are known to offer the advantage of higher yields when used in suspension cultures. Since the advent of microcarrier technology, a number of materials have been used as microcarriers (20–22). Synthetic hydrogels have received little attention as candidates for microcarrier culture. Apart from higher yield, another advantage of microspheres relates to the ease with which cells grown on them can be transferred from one culture vessel to another without the use of proteases or chelators that alter the integrity of the plasma membrane. Further, in kinetic studies, microspheres bearing the cells can be withdrawn from the culture vessel at different intervals of time, and assayed.

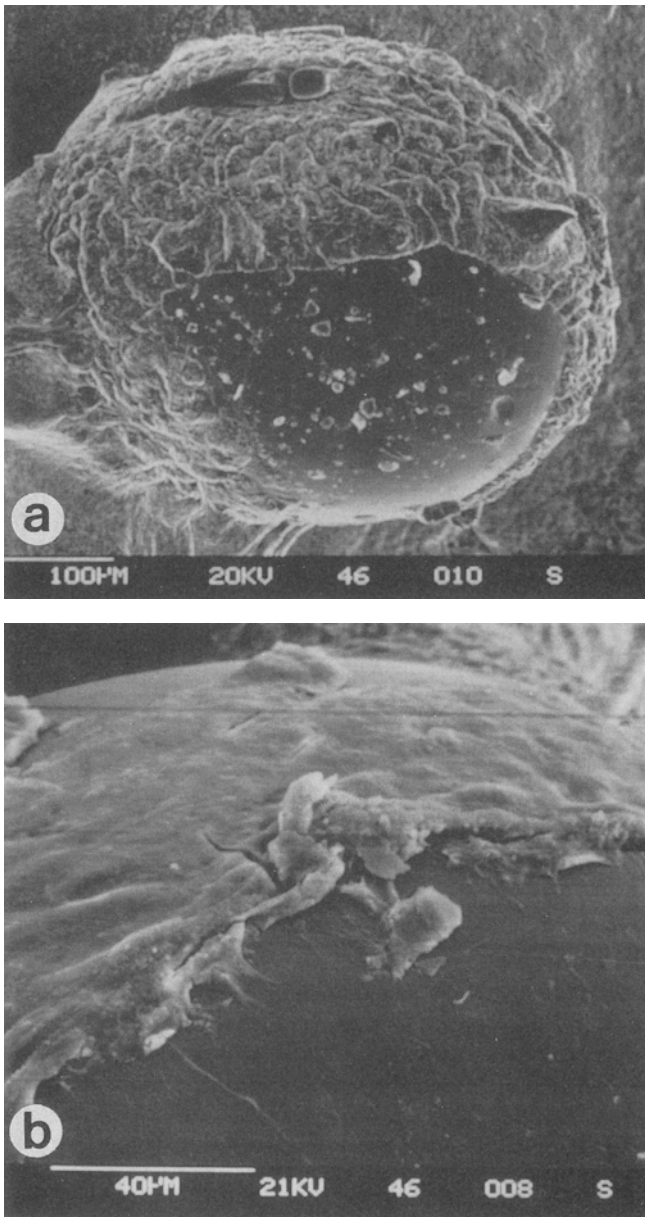

FIG. 3. Scanning electron micrographs of human lung cells grown on the hydrogel microspheres.

TABLE 1

[^{14}C]AMINO ACID UPTAKE AND INCORPORATION INTO CELLULAR PROTEINS. A COMPARISON OF HUMAN SKIN FIBROBLASTS GROWN ON HYDROGEL MICROSPHERES AND TISSUE CULTURE PLATES[a]

Culture surface	Uptake, cpm/mg Protein	Acid Precipitable Counts, cpm/mg Protein
Tissue culture plate	429668	21612
Hydrogel microsphere	443982	22768

[a]Experiments were carried out as described under Materials and Methods. Results of a representative experiment (one of four performed) are given.

It is now well known that many important cellular characteristics are influenced, to a great extent, by specific signals received at the cell surface (4). The molecular mechanisms underlying the transduction of such signals into a biological response are only beginning to be understood. The development of growth-supportive surfaces is one of the important approaches to the understanding of such interactions at the cell surface. Further, growth-supportive surfaces, when derivatized with molecules of biological interest, provide invaluable insights into the mechanisms underlying the phenomenon of cell adhesion (2,9,11,17,18) and furnish information regarding the role of specific glycoproteins or glycolipids in cell attachment (3,16). The work described in this communication represents a step toward understanding such interactions at the cell surface. Brandley et al. (4) have outlined various derivatization schemes for introducing chemical functional groups into polyacrylamide gels. Having developed a surface that is generally supportive of cell growth, we propose to derive chemically defined surfaces along similar lines to study the regulation of cellular behavior by signals received at the cell surface.

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