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Controlled Release of Epidermal Growth Factor (EGF) from EGF-loaded Polymeric Nanoparticles Composed of Polystyrene as Core and Poly(methacrylic acid) as Corona *in vitro*

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Polymeric nanoparticles composed of polystyrene (PS) as core and poly(methacrylic acid) (PMA) as corona were prepared by the dispersion copolymerization. The potential of the nanoparticles as carriers for recombinant human epidermal growth factor (EGF) was investigated. The nanoparticles showed monodispersity and good water-dispersibility. The loading content of EGF to the nanoparticles was very high due to electrostatic interaction between EGF and nanoparticles. EGF was released as a pseudo-zero order pattern after initial burst effect. The nanoparticles were sufficient for A431 cells proliferation.

Key words: Polymeric nanoparticles, Recombinant human epidermal growth factor, A431 cells, Cell proliferation

INTRODUCTION

Growth factors can either stimulate or inhibit cellular proliferation, differentiation, migration, adhesion and gene expression (Stocum, 1997). Growth factors initiate their action by binding to specific receptors on the surface of target cells. Expansion of growth factors through recombinant technologies, improved understanding of their functions and clinical applications have increased the need for tissue engineering (Babesee *et al.*, 2000). However, a short biological half-lives of growth factors, their large size, slow tissue penetration and their potential toxicity at systemic levels have many limitations in conventional routes of administration. Thus, it is required to enhance their activities *in vivo* to apply growth factors in tissue engineering. One way of enhancing the in vivo efficacy of growth factors is to facilitate the controlled release of growth factors over an extended time period by their incorporation into a polymer carrier. Through incorporation into polymer carrier, structure of growth factors and biological activity can be stabilized, prolonging the length of time over which activity is released at the delivery site (Babensee *et al.*, 2000). Among the growth factors, epidermal growth factor (EGF), a smaller 6.4 kDa protein, is sometimes considered as a hormone since it circulates in the blood, and may therefore, act on a number of different cell types (Pimentel, 1994). In culture, EGF promotes short term DNA synthesis and proliferation of chondrocytes (Vivien *et al.*, 1990) and a decrease in type II collagen expression (Jakob *et al.*, 2001). EGF initiates multiple cellular responses including auto-phosphorylation of its own receptor, generation of inositol phosphates, increasing intracellular calcium and initiation of cell mitosis (Tarnawski *et al.*, 1998). Poly(D,L-lactic-co-glycolic acid) microparticles containing EGF co-transplanted with hepatocytes in poly(vinylalcohol)-coated poly(L-lactic acid) scaffolds enhanced engraftment of hepatocytes (Mooney *et al.*, 1996).

In this study, we want to evaluate polymeric nanoparticles composed of polystyrene as core and poly(methacrylic acid) (PMA) as corona for controlled release of EGF *in vitro*. In previous studies, the polymeric nanoparticles were useful as the carriers for peptide drugs (Sakuma *et al.*, 1997; 2002).

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MATERIALS AND METHODS

Materials

Nanoparticles were prepared and characterized by previous procedures reported in Akashi *et al.*'s earlier article (Akashi *et al.*, 1990). Synthesis scheme of nanoparticles is shown in Fig. 1. Recombinant human EGF was a gift from Hitachi Chemical Co. (Tokyo, Japan).

Measurement of dynamic light scattering (DLS) and zeta potential

The particle sizes and surface charge of nanoparticles were assessed using an electrophoretic light scattering spectrophotometer (ELS 8000, Otsuka Electronics, Osaka, Japan).

Observation of nanoparticles by scanning electron microscopy (SEM)

A drop of nanoparticles was placed on a stud. After air-drying at room temperature, the sample was coated with gold using a JEOL JFC-110E Ion Sputtering device (Japan). SEM observation was made using JSM 5410LV scanning electron microscope (JEOL, Japan).

Preparation of EGF-loaded polymeric nanoparticles

EGF loading was carried out as follows: 100 ng EGF dissolved in 500 phosphate buffered saline (PBS 0.1 M, pH 7.4) was added to 1 mg nanoparticles dispersed in 500 μL PBS. The solution was stored at 4°C for 24 h. Then, the mixture of EGF and nanoparticles were centrifuged to separate the EGF that was not bound with nanoparticles, from the EGF incorporated in nanoparticles. EGF loading content was obtained by ELISA assay method.

In vitro release

The release experiment *in vitro* was performed as follows: 100 μg of EGF loaded-polymeric nanoparticles and 1 mL of PBS were put into a eppendorf tube and then this was incubated in shaking bath at 37°C. At specific time intervals, the whole medium was taken and replaced with fresh PBS. The released amounts of EGF was determined by EGF-ELISA.

Cell culture

A431 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS.

DNA synthesis

A 8×10^3 cells/0.1 mL of A431 cells were placed into 96-well PS dish precoated with collagen solution (5 mg/mL in 0.01 N HCl) for 3 h. Then EGF-loaded nanoparticles were added to the above dishes and incubated at 37°C in a humidified air/CO_2 (95/5 vol.%) incubator. DNA synthesis was performed by the incorporation of 5-bromo-2'-deoxyuridine (BrdU). The incorporation of BrdU was measured by the cell proliferation ELISA system version 2.

RESULTS AND DISCUSSION

Fig. 1 shows the synthesis of nanoparticles. This method is divided into three processes that is radical oligomerization of monomers initiated by 2,2'-azobisisobutyronitrile (AIBN) in the presence of 2-mercaptoethanol (2M EtOH) to give oligomers terminating in hydroxyl groups, the condensation of the oligomers and *p*-chloromethylstyrene (*p*-CMSt) in the presence of tetrabutylphosphonium bromide (TBPB) to give macromonomers terminating in vinylbenzyl groups, and the dispersion copolymerization initiated by AIBN between hydrophilic macromonomers and styrene. Polyanionic nanoparticles were prepared by the hydrolysis of esters located on the nanoparticle surface using HCl at 80°C for 24 h. Monodispersed nanoparticles exhibited good water-dispersibility because hydrophilic macromonomer chains were located on the surface of hydrophobic PS nanoparticles. The morphology of nanoparticles observed by SEM was shown in Fig. 2. It was observed as the spherical shapes. The sizes of nanoparticles measured by DLS were around 383 nm which was similar to SEM observation. And the surface charge of the nanoparticles was -81.8 mV, indication of polyanionic nanoparticles.

The loading content of EGF to the nanoparticle was 100 wt-%. pI value of EGF is 4.6 (Hommel *et al.*, 1992), indication of acidic peptide. The high degree of EGF incorporated in this charged nanoparticles probably results from an electrostatic interaction between EGF and the macromonomer chains on the nanoparticle surface. Cumulative release of

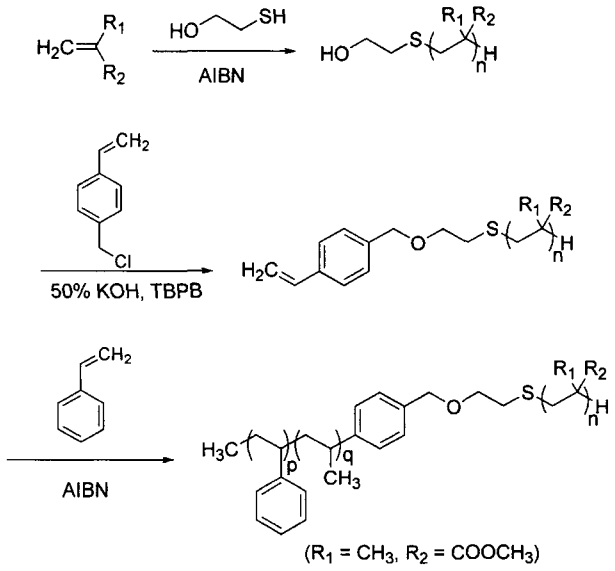

Fig. 1. Synthetic scheme of nanoparticles

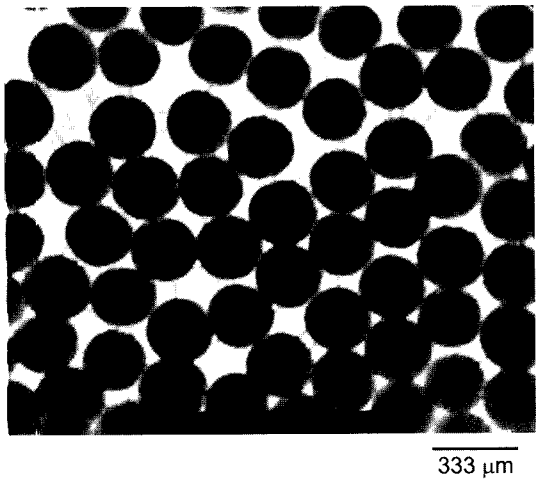

Fig. 2 Scanning electron micrograph of nanoparticles

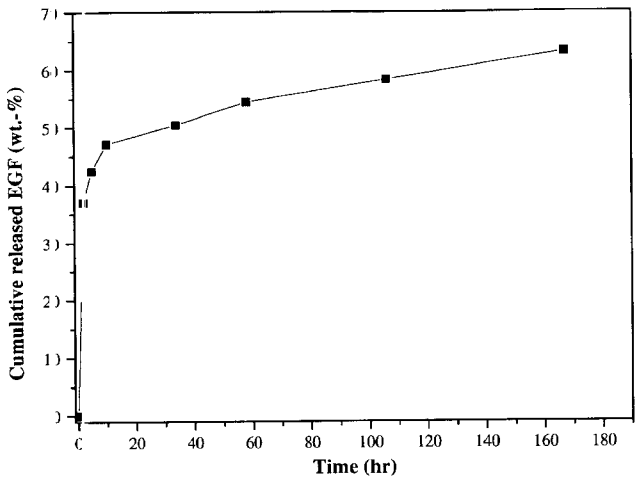

Fig. 3. Cumulative release of EGF from EGF-loaded nanoparticles *in vitro*

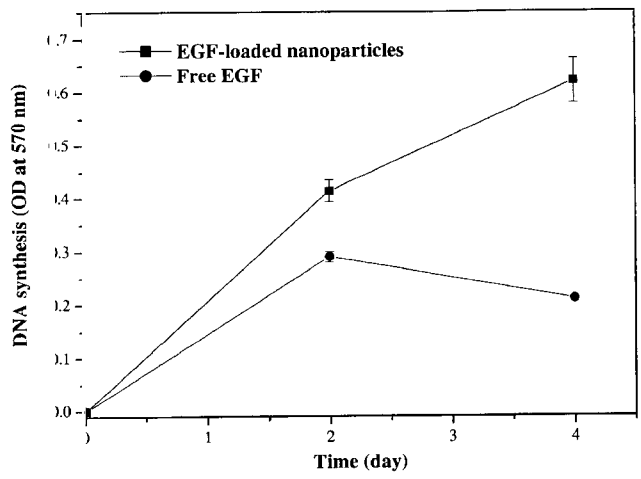

Fig. 4. DNA synthesis of A431 cells cultured on collagen-coated PS dish by released EGF from the nanoparticles and by free EGF

EGF from nanoparticles *in vitro* was shown in Fig. 3. The results indicated that EGF was released as a pseudo-zero order pattern after initial burst effect due to ion exchange mechanism in release of EGF. The cumulative amount of EGF for 167 h was 63.1 ng. Considering that a relatively small amount of EGF is needed to stimulate cell proliferation, the released amounts from nanoparticles over the extended time period are expected to be sufficient for cell proliferation (Gill *et al.*, 1981). As a matter of fact, proliferation of A431 cells cultured on collagen-coated PS dish by released EGF from the nanoparticles was shown in Fig. 4. The results indicated that the DNA synthesis was more stimulated by released EGF from nanoparticles than by free EGF. It is thought that the controlled release of EGF from the EGF-loaded nanoparticles continuously stimulated the cell proliferation than the free one.

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