Redox-active cytocompatible phospholipid polymer hydrogels for three-dimensional electrical control of encapsulated living cells

Xiaojie Lin1, Tomohiro Konno2, and Kazuhiko Ishihara1,2

1Department of Materials Engineering, 2Department of Bioengineering,
School of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
E-mail: lin@mpc.t.u-tokyo.ac.jp

Three-dimensional encapsulation of a living human cervical cancer HeLa cell in a redox-active cytocompatible phospholipid polymer hydrogel and the regulation of cell functions with simple bioelectrical stimulation were investigated. A redox-active and cytocompatible polymer based on the water-soluble phospholipid polymer, poly(2-methacryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)-co-p-vinylphenylboronic acid (VPBA)-co-vinylferrocene (VFc)) (PMBVF) was synthesized by conventional radical polymerization method. A HeLa-Fucci cell, a human cervical cancer HeLa cell stably expressing fluorescent ubiquitination-based cell cycle indicator (Fucci) were cultured and suspended into the medium, in which a PMBVF polymer were pre-dissolved with desired concentration. Poly(vinyl alcohol) (PVA) solution was added to this PMBVF/HeLa-Fucci medium solution with gently mixing at room temperature, in which case, a reversible PMBVF/PVA hydrogel with encapsulated HeLa-Fucci cells was obtained. The morphologies of the PMBVF/PVA hydrogel and the three-dimensional encapsulated HeLa-Fucci cells were confirmed with microscopy. Cyclic voltammograms results indicated that PMBVF/PVA hydrogel still had excellent electrochemical properties even in the presence of living HeLa-Fucci cells. A constant voltage (0.4 V vs. Ag/AgCl) was applied to the extracellular environment of encapsulated cells as electrical stimulation and the cell cycle progression was calculated base on the analysis of fluorescent images. Compared with that without electrical stimulation, a delay of the cell cycle progression could be observed between 8 h and 18 h while the cells were encapsulated in the hydrogel with electrical stimulation. The investigation of cell functions based on a three-dimensional electrical stimulation with a redox-active cytocompatible phospholipid polymer hydrogel will promote the development of electrochemical therapy of cancer and tumor.

Keywords: Redox polymer, phospholipid polymer hydrogel, cytocompatibility, electrical stimulation, cell proliferation cycle

1. INTRODUCTION
Bioelectricity stands for the potential and polarity changes of biological organs, tissues and cells in biological processes. It is the performance of normal physiological activity, and an essential feature of biological activity of living organism, that is always accompanied by the changes of cell membrane potential and electron transfer inside the cells. Thus living cell behavior and bioelectricity has very closely and electron transfer inside the cells. Therefore, redox-active cellular matrix-like cytocompatible polymer hydrogel will be attractive materials for investigation of living cell responsive behavior to the bioelectrical stimulation, further to promote the development of electrochemical therapy.

MPC polymers contain extremely hydrophilic phosphorylcholine groups and have a cell membrane-inspired structure that prevents the denaturation of proteins. The MPC polymers have been designed that are of diverse structures, such as particles, fibers, hydrogels, colloids, and brush surfaces. In the previous study, we have prepared a redox-active water-soluble and amphiphilic MPC polymer with a phenylboronic acid moiety and a electron transfer moiety, poly(2-methacryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)-co-p-vinylphenylboronic acid (VPBA)-co-vinylferrocene (VFc)) (PMBVF) [8]. The PMBVF can
form a hydrogel with PVA in an aqueous cell culture medium at room temperature, this hydrogel has been proven not only has excellent electrochemical properties, but also provides a mild environment for living cells, and it maintains cellular function for long periods.

In this research, encapsulation of a living human cervical cancer HeLa cell in a redox-active cytocompatible phospholipid polymer hydrogel (PMBVF/poly(vinyl alcohol) (PVA) hydrogel) and the cell cycle progression after three-dimensional electrochemical stimulation were investigated (Figure 1). The detail cell behaviors in the presence or absence of 3D electrochemical stimulation based on this redox-active cytocompatible phospholipid polymer hydrogel were discussed.

2. EXPERIMENTAL
2.1 Materials
MPC was obtained from NOF Co., Ltd., (Tokyo, Japan), where it was synthesized by a previously reported method [9]. BMA, VPBA, VFc, PVA (average polymerization degree 1000, completely hydrolyzed) and β-D-Sorbitol (sorbit) were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). 2,2'-Azobisisobutyronitrile (AIBN) was purchased from Kanto Chemicals Co., Inc. (Tokyo, Japan). Cell culture medium and its supplements (Dulbecco’s modified Eagle’s medium (DMEM) with/without phenol red, fetal bovine serum (FBS), trypsin (TrypLE express) and ultrapure distilled water) were purchased from Invitrogen (NY, USA). Thymidine was purchased from Sigma (MO, USA). The other organic reagents and solvents were commercially available reagents of extra-pure grade and were used without further purification.

2.2 Preparation of the PMBVF/PVA hydrogel
The PMBVF was synthesized by a conventional radical polymerization as previously described [8, 10]. Briefly, MPC (15 mmol), BMA (6.0 mmol), VPBA (4.5 mmol), VFc (4.5 mmol) and AIBN (0.3 mmol) were dissolved in 30 mL of ethanol and the mixture was deoxygenated with argon gas in a sealed flask prior to polymerization at 60 °C for 72 h. Once formed, the polymer was purified by reprecipitation using a mixed solvent of ether/chloroform (90/10 v/v). The polymer was filtered off and collected as a yellow powder after vacuum desiccation, followed by dialysis against distilled water and freeze-dry. Chemical structures of the polymers were confirmed using 1H NMR. The average molecular weights of the polymers were measured using a gel permeation chromatography (GPC) system (JASCO, Tokyo, Japan) in water/methanol (30/70 v/v) solution containing 10 mmol/L of lithium bromide. Poly(ethylene oxide) was used as the standard for the calibration curve. The chemical structure of PMBVF and the hydrogel formation mechanism are shown in Figure 2. The obtained PMBVF polymer and PVA were dissolved into the ultrapure distilled water with 5.0 wt% concentration, respectively. These two kinds of polymer solution were blended together with various ratios to prepare the PMBVF/PVA hydrogel. The obtained hydrogels were freeze-dried, followed by the observation of three-dimensional morphology with a scanning electron microscope (SEM, Topcon, Tokyo, Japan).

2.3 Cell culture
A human cervical cancer cell line expressing a fluorescent ubiquitination-based cell cycle indicator (HeLa-Fucci; RCB2812, RIKEN Bio-Resource Center) was seeded into a conventional cell culture dish (5 x 10^4 cells/mL) in DMEM (with phenol red) supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO2 [11]. Sub-confluent cells were passaged after trypsin digestion.

2.4 Encapsulation of living cells in hydrogel
The HeLa-Fucci cells were suspended into the PMBVF/DMEM solution with desired concentration. PVA/DMEM solution was added to this PMBVF/HeLa-Fucci medium solution with gently mixing at room temperature, in which case, a reversible PMBVF/PVA hydrogel with encapsulated HeLa-Fucci cells was obtained. The morphologies three-dimensional encapsulated HeLa-Fucci cells in the hydrogel were observed with a phase-contrast microscopy (model IX 71; Olympus, Tokyo, Japan).

2.5 3D electrical stimulation
An electrochemical analyzer (ALS/CH Instrument, Tokyo, Japan) was used to perform the measurement of electrochemical property and electrical stimulation. The electrochemical cell, as a reactor, is a single-chamber
three-electrode system. Tin-doped indium oxide (ITO) grown on a glass substrate by spray pyrolysis deposition was used as the working electrode (WE) on the bottom surface of the reactor; the counter electrode (CE) and the reference electrode (RE) were Ag/AgCl (KCl saturated) and a platinum wire, respectively.

HeLa-Fucci cells were arrested using the double thymidine block method to obtain synchronized cells [12]. PMBVF/DMEM (no phenol red) solution (5.0% w/v, \(5 \times 10^5\) cells/mL) 0.8 mL was gently mixed with 0.8 mL PVA/DMEM (no phenol red) solution (5.0% w/v) in the electrochemical cell. The cyclic voltammetry (CV) measurements of this PMBVF/PVA hydrogel with encapsulated living cancerous cells was evaluated at a scan rate of 50 mV/s at 37 °C in a humidified atmosphere containing 5% CO2. An external applied potential 0.4 V (vs. Ag/AgCl, 10 min) was added under a potentiostatic condition one time every 2 h for 12 times. The fluorescent images of cells encapsulated in the hydrogel were obtained with the fluorescent mode of a phase-contrast microscopy alternated with stimulation, one time every 2 h for 12 times. In order to eliminate the effect of fluorescence, the normal HeLa cells were used instead of HeLa-Fucci cells in the hydrogel. After electrical stimulation, the hydrogel were dissociated with sorbitol/DMEM solution (1 mol/L). Cells were obtained after centrifugation and rinse with DMEM. Then, cell viability was visually assessed using a live/dead kit (L-3224; Invitrogen). Fluorescence images were captured using a fluorescence microscope and the numbers of live and dead cells were counted to quantify cell viability.

2.6 Statistical analysis of the data
All graphs and bar charts are expressed as the mean ± SD of triplicate repeated experiments. Student’s t test was carried out to determine whether the observed differences were statistically significant (\(p < 0.05\)).

3. RESULTS AND DISCUSSION
3.1 Morphology of the hydrogel and encapsulated cells
The PMBVF was synthesized through a conventional polymerization method which is similar to one previously reported [8]. The chemical structure is shown in Fig. 2A. The MPC/BMA/VPBA/VFc unit composition in polymer was 52/23/15/10. This result also indicates that PMBVF is a water-soluble polymer due to its high composition of MPC units (52 % mole fraction). When PMBVF and PVA aqueous solutions are mixed together, the PMBVF/PVA hydrogel is formed spontaneously via a covalent cross-linking process between the phenylboronic acid groups of the VPBA units in PMBVF and hydroxyl groups in PVA (Fig. 2B).

Fig. 3 shows the morphology of hydrogel and the HeLa-Fucci cells encapsulated in the hydrogel. The 3D structure of the hydrogel can be observed from Fig. 3A. The blend ratio of the PMBVF/PVA according to the volume is 1/1. The size of the porous structure could be controlled through changing the concentration of the polymer solution and the blending ratio of polymer solution, to prepare hydrogel with different physical mechanical properties. Fig. 3B shows images of 3D encapsulated HeLa-Fucci cells in the PMBVF/PVA hydrogel, where round shape of cells still be maintained after encapsulation. This result is absolutely different from those cells being cultured in DMEM in a normal cell culture dish, which tend to adhere, spread, and flatten to the surface and exhibit heterogeneous morphologies.

Figure 3. (A) Scanning electron microscope images of the PMBVF/PVA hydrogel; (B) phase-contrast microscope image of 3D encapsulated HeLa-Fucci cells in the hydrogel.

Figure 4. Cyclic voltammograms of the PMBVF/PVA hydrogel in the presence of cells (solid line) or absence of cells (dash line).

3.2 Electrochemical property
Fig. 4 shows the CV results of the PMBVF/PVA hydrogel in the presence of cells (solid line) or absence of cells (dash line) at a scan rate of 50 mV/s at 37 °C in a humidified atmosphere containing 5% CO2. Initially, the VFc units in the PMBVF contained only the reduced form of iron, Fe(II). The Fe(II) is oxidized to Fe(III) during the forward scan from 0 V to 0.6 V, and an oxidation current peak is observed. Then, Fe(III) is reduced to Fe(II) during the reverse scan from 0.6 V to 0 V, and a reduction current peak is observed. This demonstrates that the PMBVF/PVA hydrogel can act as a mediator of electron transfer. Both the oxidation and reduction peaks decreased after the cells were encapsulated in the hydrogel comparing to the original hydrogel, this may be because the lipid bilayer of cell membrane blocked the transferring of electrons between ferrocene group in the hydrogel.

3.3 Three-dimensional electrical stimulation
After the encapsulation of the HeLa-Fucci cells in the PMBVF/PVA hydrogel, an external applied potential
0.4 V (vs. Ag/AgCl, 10 min) was added under a potentiostatic condition one time every 2 h for 12 times. In order to eliminate the effect of fluorescence from HeLa-Fucci cells to measure the cell viability, the normal HeLa cells were used to instead of HeLa-Fucci cells in the hydrogel. Both in the presence or absence of external applied potential, more than 80% of the cell viability could be maintained (Fig. 5). That mean, the PMBVF/PVA hydrogel can maintain the function of encapsulated cells. Similar results were obtained in another study, in which a bacteria, Shewanella were encapsulated in the hydrogel and high viability could be maintained with normal cellular metabolism [8].

4. CONCLUSIONS
A redox-active cytotocompatible phospholipid polymer hydrogel (PMBVF/PVA hydrogel) acted as a cytotocompatible cellular matrix for spontaneous and reversible encapsulation of living cells. Also it functioned well as a mediator of electron transfer. A delay of the encapsulated cancerous cell cycle progression was observed after 3D electrical stimulation. This hydrogel provide an extracellular matrix-like platform to perform the first study of living cells behavior in a 3D cellular matrix-like environment through electrical stimulation, further to provide us more comprehensive and actual information for clinical applications.

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