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Design of Two Complementary Copolymers Work as a Glue for Cell-Laden Collagen Gels

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We designed two types of copolymers that play a role of "polymeric glue". They introduced surface adhesive functions to cell-laden collagen gels. The present study realised surface functionalisation of naturally derived hydrogels and suggested a novel method for manupilating living cells. This method is potentially useful for *in vitro* reconstruction of 3D living tissue since it enabled versatile and cytocompatible hydrogel surface modification by a simple procedure without any special equipment.

Hydrogels, which are composed of 3D polymer networks and water as solvent, have unique surface properties such as molecular diffusivity, low friction, high viscosity, high wettability, and antifouling properties. Owing to these properties, hydrogels have been studied for applications in artificial cartilage, contact lenses,[1] sensor devices,[2–4] tissue engineering,[5–7] and so on. Moreover, in recent years, "designing" of surface properties of hydrogels such as repellence,[8,9] adhesion,[10] and diffusivity,[11,12] has begun. They have provided novel insights into applications of hydrogels. However, this strategy has been limited to synthetic hydrogels so far. Hydrogels composed of natural polymers such as fibrin, Matrigel™, and collagen gel also need improvement in surface properties.

Natural hydrogels are useful as cell culture materials because they can encapsulate cells, have ECM-like elasticity,[13] and include important endogenous factors of living tissues.[14] To precisely mimic the environment inside a living body, a 3D co-culture system using hydrogels has been the focus of attention. This system can offer environments not only for cell-ECM interactions, but also cell-cell interactions consisting of more than two types of cells. Using this method, we can reconstruct micrometre to millimetre scale 3D living tissues composed of different types of cells *in vitro*. Noteworthy, just mixing two types of cells can not realize reconstruction of spatially ordered tissues because the same type of cells tends to aggregate between themselves because of the cadherin's property, which limits cell-cell interaction between different types of cells.[15–17] This means that special methods are required to realize spatially ordered reconstruction. For example, cell-cell direct connection via DNA coated onto the cells has been reported to realize cell arrangement.[18] Although this technique was great, it was invasive because it chemically modified cell surface. Using hydrogel, we can arrange intact cells. For hydrogel arrangement, spatial arrangements of cell-laden hydrogels using their "key and keyhole" shapes,[19] moulding of micron-sized gel beads,[20] and spatially homogeneously distributed molecular recognition units have

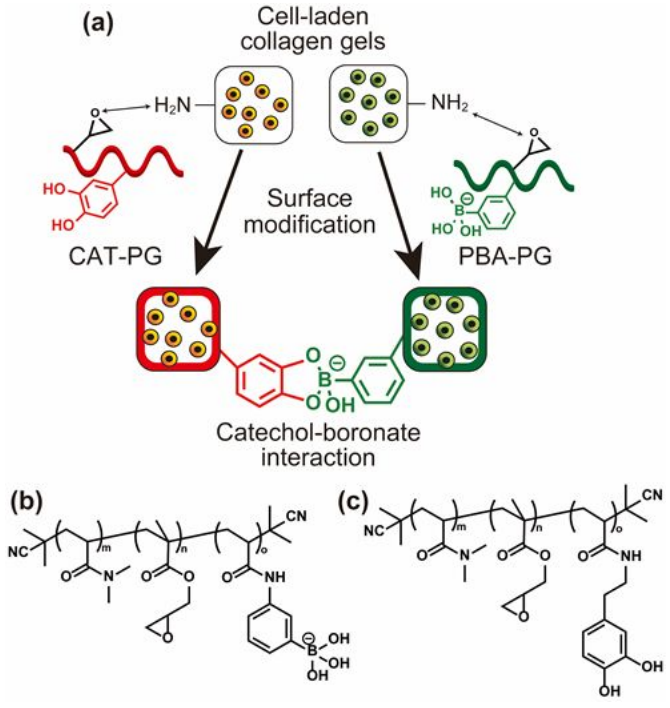

Fig.1 (a) Conceptual illustration of the gel-to-gel adhesion system using designed copolymers, PBA-PG and CAT-PG. Chemical structures of (b) PBA-PG and (c) CAT-PG.

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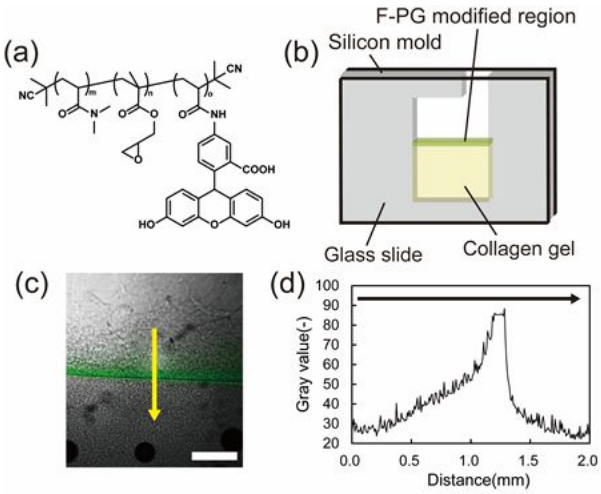

Fig.2 (a) Chemical structure of a fluorescent copolymer, F-PG. (b) Illustration of the experimental setup for analysing spatial distribution of F-PG on a collagen gel. (c) Merged image from fluorescent and optical images of F-PG-modified collagen gel. Scale bar = 1 mm. (d) The distribution of F-PG intensity from fluorescent image on yellow line shown in (c).

been suggested.[21] However, they continue to have some shortcomings, including low cell viability, difficulty of treatments, and limitation of cell types. Under the circumstances, we conceived to develop a novel "surface designing" method for naturally derived hydrogels to reconstruct spatially ordered living tissue. Specifically, we introduced molecular recognition units onto the surface regions of cell-laden collagen gels to add the property of complementary gel-to-gel adhesion.

In this study, to develop a versatile and cytocompatible method, we fabricated two types of copolymers that could be modified onto cell-laden gels. The copolymers induce gel-to-gel adhesion without damaging the encapsulated cells (Fig.1). The copolymers are consisting of three monomers: a hydrophilic monomer, a monomer having an epoxide group that can reacts with collagen gels,[22] and a monomer with a molecular recognition unit, which is phenyl boronic acid (PBA) or catechol (CAT). The modification procedure involves just immersing collagen gels into the Dulbecco's phosphate-buffered saline (D-PBS) containing the polymeric glue. This method enables the cytocompatible "*in situ*" surface modification of any type of cell-laden gel by a simple procedure without requiring any special equipment. We suggest that this should be a good strategy for realising safe and realistic tissue engineering.

First, two types of copolymers, PBA-PG and CAT-PG, composed of *N,N*-dimethylacrylamide (DMAAm), glycidyl methacrylate (GMA), and 3-acrylamide phenyl boronic acid (APBA) or dopamine acrylamide (DA), were synthesised *via* free radical polymerisation. The ^{1}H-NMR charts have been shown in Fig. S1, ESI†.

To confirm the complementary interaction between PBA-PG and CAT-PG, the Dulbecco's Modified Eagle Medium (DMEM-LG) solution of each copolymer was mixed with each other. As a result, the solution rapidly increased in viscosity after mixing (Fig.S2, ESI†). This phenomenon has been observed in studies using the complementary interaction of synthetic polymers and has been estimated to be caused by boronate ester formation.[23] From this result, it was shown that the PBA and CAT sites in these copolymers could interact with each other at the physiological pH in the presence of 1 g/L of glucose. PBA is known to form a reversible bond not only with catechol but also with glucose.[24,25] However, the previously reported association constant between catechol and phenylboronic acid is much larger than that between PBA and glucose over a wide range of pH values.[26]

To confirm the modification of copolymers onto the surface regions of collagen gels, the fluorescent copolymer, F-PG, which has a composition similar to polymeric glue, was used (Fig.2(a)). F-PG solution diluted with D-PBS was poured onto a collagen gel synthesised in the mould as shown in Fig.2(b), and it was kept for 30 min to modify F-PG onto the gel. After dialysis with D-PBS, the outermost surface of the gel, which was attached to the F-PG solution, was observed using a fluorescent microscope. Fig.2(c) shows the overlay of the fluorescent image on the visible image. From this result, the fluorescent region appears to be concentrated on the outermost surface of the gels. The fluorescence range was approximately 200 µm, as estimated by luminance analysis with Image J (Fig.2(d)). This may be caused by the low diffusion rate of F-PG against the hydrogel because it becomes more difficult for larger molecules to penetrate the 3D polymer network. For instance, the diffusion coefficient of lysozyme in gelatin hydrogels is 10 to 100 times lower than the diffusion coefficient of the protein in the water.[27,28]

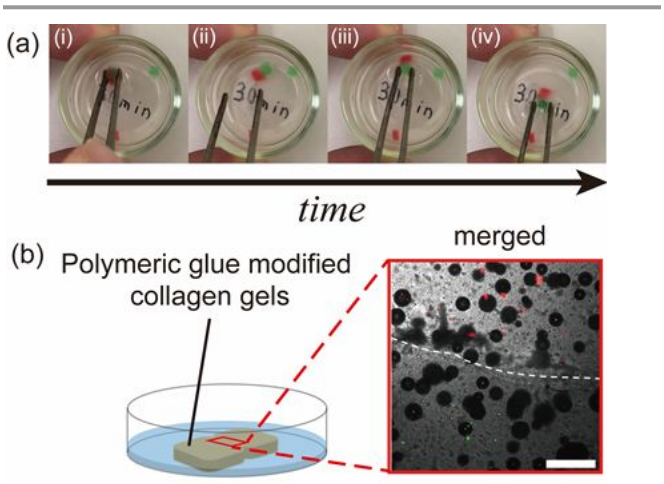

Fig.3 (a) Sequential images explaining the procedure of the gel-to-gel adhesion experiment between the PBA-PG-modified collagen gel (green) and the CAT-PG-modified collagen gel (red). (i) Gels were attached to each other manually using a tweezer. (ii) Gels were kept in their adhesive state even after releasing the tweezer. (iii) Then, the green gel was grabbed by a tweezer and moved slightly. (iv) The red gel was moved along with the green gel maintaining the gel-to-gel adhesion. (b) Illustration and image of adhesion of cell-laden collagen gels via interaction between PBA-PG and CAT-PG. Merged image from fluorescent and optical images of the border region of adhered cell-laden gels has been shown in the red square. Hep G2 cells encapsulated in CAT-PG-modified collagen gel and NIH/3T3 cells encapsulated in PBA-PG-modified collagen gel were stained with red and green, respectively. Scale bar = 500 µm.

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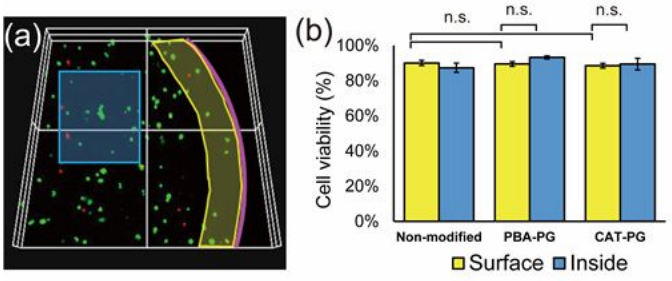

Fig.4 (a) A 3D image of stained cells in a collagen gel. Pink line indicates the edge of the gel. Blue and yellow regions have been defined as inside and surface regions of collagen gel, respectively. (b) The viability of encapsulated cells in the surface (yellow) and inside (blue) regions of non-modified, PBA-PG-modified, and CAT-PG-modified gels after 24 h encapsulation (n=3). n.s., not significant; $P < 0.05$.

The surface-modified collagen gels were then floated on D-PBS in a petri dish and contacted manually using a tweezer. After contacting the gels, one of the adhered hydrogels was dragged manually. As a result, gel-to-gel adhesion was confirmed between PBA-PG - and CAT-PG-modified collagen gels (Fig.3(a) and Movie S1, ESI†). In contrast, the same types of copolymer modified gels did not adhere to each other (Movie S1, ESI†). This meant that the specific molecular recognition between surface-modified PBA-PG and CAT-PG occurred, which induced the successful gel-to-gel adhesion. This adhesion was also observed in DMEM-LG and cell culture medium, and the molecular recognition was strong enough to allow hydrogels to adhere for more than 1 day in the presence of 1 g/L of glucose. Furthermore, it is noteworthy that hydrogel adhesion also occurred in the case of Hep G2-laden and NIH/3T3-laden collagen gels (Fig.3(b)).

Finally, to investigate cytotoxicity of the encapsulated cells, cell viability was confirmed by counting live and dead cells separately. Hep G2 cells dispersed in the pre-gel solution were converted to collagen gels on glass bottom dishes. After the surface modification of Hep G2 cell-laden collagen gels, they were incubated in cell culture medium for 24 h. Then, the encapsulated cells were stained with calcein-AM and ethidium homodimer (live/dead dyes) and observed using a confocal laser microscope. The viability of encapsulated cells was calculated by counting live and dead cells, separately, and compared between regions within 200 μm from the edges of the hydrogels (surface regions) and the other regions (inside regions) (Fig.4(a)). The viability of Hep G2 cells encapsulated inside PBA-PG-modified and CAT-PG-modified collagen gels and non-modified ones were compared. Specifically, the viability of cells in the surface and inside regions of the three gel samples were compared (Fig.4(b)). From the results, there was no significant difference observed among these conditions and cell viability was kept at high level (about 90%). Thus, we concluded that the copolymers modified on the collagen gels did not show apparent cytotoxicity to the encapsulated Hep G2 cells. High concentrations of PBA and quinone groups derived from CAT often work as cytotoxic factors.[29,30] In particular, CAT is easy to convert to quinone groups and shows strong toxicity. In a previous study, we developed synthetic hydrogels containing PBA and CAT homogeneously distributed in 3D cell culture. In this case, the viability of cells encapsulated in the CAT-containing gel is low (about 50%) even in the presence of a reducing agent.[21] In contrast, in the present study, we successfully reduced the cytotoxicity of CAT owing to the surface-localised introduction of copolymers.

In conclusion, we successfully realised cytocompatible "*in situ*" surface functionalisation of cell-laden collagen gels by simple surface modification using designed copolymers. We believe this simple, versatile, and cytocompatible method will be a strong tool for surface functionalisation of natural hydrogels, and for applications in tissue engineering and regenerative medicine. Moreover, it can be expected to develop even as a surface functionalisation method for living cells, since surface modification only requires amino groups on the surface regions of substances. It can also be expected that it may not only add adhesion property but also add different surface properties such as controlled permeability and low friction to hydrogels and other water-holding substances.

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Conflict of interest: There are no conflicts to declare.

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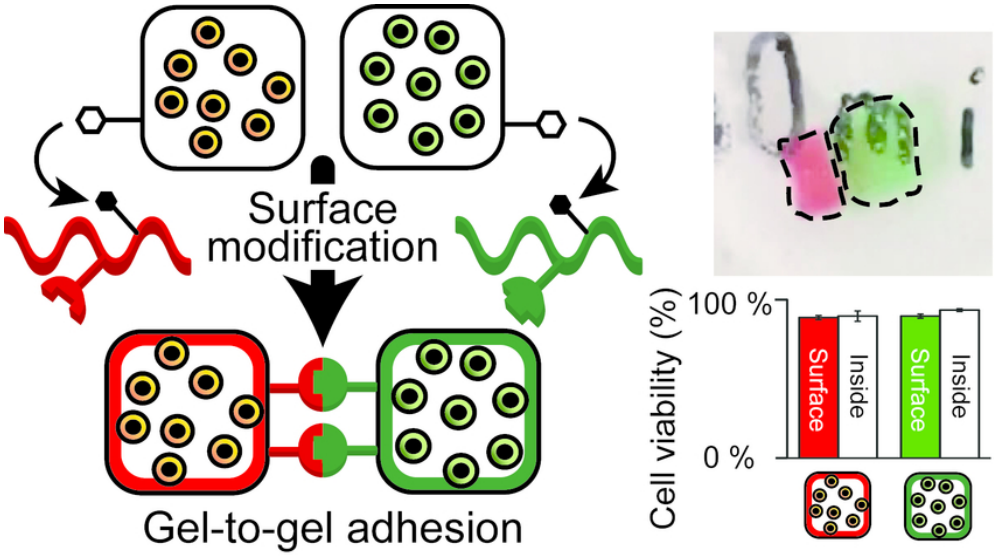
This study realized cytocompatible “in situ” surface functionalization of collagen gel for adding the property of gel-to-gel adhesion.



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