



## Original Article

# Improving chondrocyte harvests with poly(2-hydroxyethyl methacrylate) coated materials in the preparation for cartilage tissue engineering



Mikako Harata<sup>a, b</sup>, Makoto Watanabe<sup>a</sup>, Satoru Nagata<sup>c</sup>, Edward Chengchuan Ko<sup>a</sup>, Shinsuke Ohba<sup>d</sup>, Tsuyoshi Takato<sup>a, b</sup>, Atsuhiko Hikita<sup>a</sup>, Kazuto Hoshi<sup>a, b, \*</sup>

<sup>a</sup> Division of Tissue Engineering, The University of Tokyo Hospital, Tokyo, Japan

<sup>b</sup> Department of Oral-maxillofacial Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

<sup>c</sup> Nagata Microtia and Reconstructive Plastic Surgery Clinic, Saitama, Japan

<sup>d</sup> Department of Bioengineering, School of Engineering, The University of Tokyo, Tokyo, Japan

## ARTICLE INFO

## Article history:

Received 30 June 2017

Received in revised form

7 August 2017

Accepted 14 August 2017

## Keywords:

Chondrocyte

pHEMA

Antifouling

Extracellular matrix

Microtia

Cell yield

## ABSTRACT

Remarkable advances have been made in cartilage regenerative medicine to cure congenital anomalies including microtia, tissue defects caused by craniofacial injuries, and geriatric diseases such as osteoarthritis. However, those procedures require a substantial quantity of chondrocytes for tissue engineering. Previous studies have required several passages to obtain sufficient cell numbers for three-dimensional and monolayer cultures. Thus, our objective was to improve the quantity of chondrocytes that can be obtained by examining an anti-fouling polyhydrophilic chemical called poly(2-hydroxyethyl methacrylate) (pHEMA). To determine the effectiveness of the chemical, pHEMA solution was applied via dip-coating to centrifuge tubes, serological pipettes, and pipette tips. The cell quantity obtained during standard cell culturing and passaging procedures was measured alongside non-coated materials as a control. A significant 2.2-fold increase of chondrocyte yield was observed after 2 passages when pHEMA was applied to the tubes compared to when non-coated tubes were utilized. The 3-dimensional chondrocyte pellets prepared from the respective cell populations and transplanted into nude mice were histologically and biochemically analyzed. No evidence of difference in matrix production for in vitro and in vivo cultures was found as well as similar proliferation rates and colony formation abilities. The use of pHEMA provides a powerful alternative method for expanding the quantity of chondrocytes harvested and handled during cell isolation and passaging to enhance cartilage tissue engineering.

© 2017, The Japanese Society for Regenerative Medicine. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Microtia, a congenital ear deformity, has been seen at the highest prevalence in male Hispanic populations, followed closely by Asian populations. Specifically, in Japan, one out of every 10,000 births succumb to microtia [1]. Despite the high incidence rate, no definite cause has been linked to the birth defect [2], limiting the

potential for preventative medicine. Thus, finding a cure for microtia has become a heavily valued target for tissue engineering.

Autologous chondrocytes have been utilized for cartilage tissue engineering [3]; however, the limited size of the tissue has constricted the cartilage salvageable for regenerative medicine. Constructs prepared with various biodegradable polymer scaffolds [4–6] require at least  $100 \times 10^6$  cells/cm<sup>3</sup> [7, 8]. To meet the demands of required cell numbers, expansion of the obtainable auricular chondrocytes through repeated passaging becomes a necessity. While repeated expansions increase cell yield, they also deteriorate cell differentiation/maturation and decrease matrix production [9]. Multiple causes for cell damage occur throughout the cell harvesting protocol. When auricular cartilage is extracted from the microtic ear of a patient, the cartilage is incubated in collagenase for a substantial duration to digest collagen-based

\* Corresponding author. Oral and Maxillofacial Surgery, Department of Sensory and Motor System Medicine, Graduate School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan. Fax: +81 3 5800 9891.

E-mail address: [pochi-tky@umin.net](mailto:pochi-tky@umin.net) (K. Hoshi).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

matrices that encapsulate chondrocytes needed for experimental use. Excessive exposure to collagenase results in cell damage, consequently limiting the number of viable cells that can be recovered and decreasing chondrogenic abilities [10]. Nevertheless, the chondrocytes that are recovered without damage must be sufficiently retained for further processing.

A variety of techniques have been implemented to minimize excessive cell loss during the cell extraction and expansion procedures, including the re-inoculation of floating cells in monolayer culture [11], and the addition of enzymes to the cell pellet to increase the effectiveness of resuspension after centrifugation [12]. However, the addition of enzymes necessitates an accrument of an additional centrifugation step within the procedure, in effect, causing potential negative impacts on cell survival.

In this study, we sought to find an alternative method for improving harvested cell quantity along with maintaining cell quality. As a widely used step in cell culturing procedures, centrifugation contributes as one of the leading uncontrolled variables for cell loss. While controlling the time and rotational centrifugal force (RCF) of centrifugation applied to a standard lab protocol, our focus was to examine the effectiveness of an antifouling chemical called poly(2-hydroxyethyl methacrylate) (pHEMA). The merits of the pHEMA bioreagent include availability, cost efficiency, minimal preparation requirements, and guaranteed safety. The versatility of this chemical has enabled it to be employed in various applications in the form of a hydrogel including soft contact lenses, membrane dialyzers, wound dressings, testicular prostheses, and vocal cord prosthesis [13–19]. The chemical pHEMA in a non-hydrogel form is a promising antifouling agent with easy applicability. In previous studies, the efficacy of pHEMA-coated vessels was examined for retinal epithelial cells and murine embryonic stem cells, while their effects on cell characteristics were not investigated [20,21]. In addition to cell concentration within suspension, medium viscosity, hydration of the pellet, and resuspension, the cell type is one of the parameters that govern the extent of adhesion to surfaces when force is applied to cells [12,22,23].

For the purpose of determining the clinical applicability of pHEMA to the field of cartilage tissue engineering, we examined the effect of pHEMA-coated vessels to human auricular chondrocytes. PHEMA was dip-coated onto centrifugation tubes, serological pipettes, and pipette tips prior to centrifugation and resuspension of chondrocyte cell suspension. The number of cells lost, and ability of extracellular matrix production were analyzed.

## 2. Materials & methods

### 2.1. Preparation of poly(hydroxyethyl methacrylate) coated materials

1.8 g of pHEMA powder (Sigma–Aldrich) was dissolved in 45 mL of 99.5% ethanol and incubated for 72 h at 60 °C. The mixture was shaken vigorously every 24 h. At the end of the 72 h, 0.5 mL of distilled water was added to complete the dissolution of the powder. The dissolved solution was filtered through 0.20 µm Millex-LG filter unit (Merck Millipore, Darmstadt, Germany) under sterile conditions. pHEMA-coated materials used in this experiment consisted of 50 mL centrifuge tubes (Sumitomo Bakelite, Tokyo, Japan), 10 mL and 25 mL serological pipettes (Sumitomo Bakelite), and 200 µL pipette tips. The application of pHEMA solution onto the materials was done by a simple dip-coating all under sterile conditions.

### 2.2. Cell isolation and culture

All procedures were approved by the Ethics Committee of the University of Tokyo Hospital (ethical permission number 622).

Human auricular chondrocytes from microtia patients were obtained from Nagata Microtia and Reconstructive Plastic Surgery Clinic in compliance with the Helsinki Principles and informed consent. The auricular cartilage was separated from perichondrium and skin under sterile conditions and minced into 1 mm<sup>3</sup> pieces and digested with 45 mL of 0.3% collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in control medium [Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St. Louis, MO) containing 1% penicillin and streptomycin (P/S) (Sigma–Aldrich)] at 37 °C for 18 h to isolate the chondrocytes. The digested suspension was filtered using a sterile 100 µm nylon cell strainer (BD Falcon, Bedford, MA). The number of cells in suspension was counted with Nucleo Counter (ChemoMetec A/S, Denmark) and centrifuged at 440 g for 5 min at 4 °C (AX-511, Tomy Seiko Co., Ltd., Tokyo, Japan). The P0 original auricular chondrocyte suspension was seeded at a concentration of  $2 \times 10^5$  cells/10 mL onto 100 mm collagen type I dish (Iwaki Co. Ltd., Tokyo, Japan) in proliferation medium [Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich) supplemented with 5% human serum (Sigma–Aldrich), 5 µg/mL insulin (Novo Nordisk Pharma Ltd.), 100 ng/mL fibroblast growth factor-2 (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan), and penicillin/streptomycin] and then cultured under monolayer conditions as P0 for 7 days at which the cells were reaching confluence. The medium was fully changed once every 3 days, and on day 7, the cultured cells were collected using trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma–Aldrich).

### 2.3. Colony formation analysis and crystal violet stain

Two hundred cells were seeded onto 100 mm (diameter) Collagen Type I dishes. Proliferation medium was used to culture the colony for 14 days. The culture medium was changed twice a week. On day 14, the dishes were fixed with 100% methanol for 10 min and stained with 0.5% Crystal Violet (Sigma–Aldrich) for 10 min. The vessels were then rinsed under running water and dried. All colonies with a diameter of >2 mm were counted.

### 2.4. Preparation of 3-dimensional (3D) pellet

To form the 3D atelocollagen constructs,  $10^7$  cells/mL chondrocyte cell suspension from the pHEMA and non-coated conditions post-centrifugation were made into 100 µL constructs containing 1% atelocollagen, which was diluted from 3% atelocollagen (Koken Co., Ltd., Tokyo, Japan). The constructs were incubated for gelation for 2 h at 37 °C in agarose molds made of 2% agarose and control medium which has a  $5 \times 5 \times 5$  mm concavity. The 100 µL pellets were extracted and cultured for 3 weeks in differentiation medium [DMEM/F-12 medium containing penicillin, streptomycin, and insulin-like growth factor-I (Astellas Pharma Inc., Tokyo, Japan)].

### 2.5. Implantation of 3D pellets to nude mice

All experiments were performed according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo. Immunocompromised BALB/cA<sub>J</sub>c1-nu/nu mice (6 weeks old, male) were obtained from Nisseizai, Tokyo, Japan. Under general anesthesia application with Isoflurane (Sigma–Aldrich), a 10 mm left dorsal skin incision was made in the nude mouse. The chondrocyte pellets cultured in vitro for 3 weeks were harvested and inserted subcutaneously. Four weeks after the implantation, the mice were euthanized, after which the pellets were harvested. Blood vessels and excess tissues were trimmed from the pellets, and photographed before being subjected to histological and biochemical analysis.

## 2.6. Histological analysis

The 3D constructs were fixed in 4% paraformaldehyde (PFA) solution at room temperature for 3 h. The constructs were then embedded in paraffin and sectioned into 5  $\mu\text{m}$  slices, using standard sectioning techniques. Sections were stained with Hematoxylin & Eosin, and Toluidine Blue.

## 2.7. Biochemical analyses

The samples were minced in 0.05 M acetic acid, and 1% pepsin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added. Then the samples were incubated at 4 °C. After 48 h of incubation, 10 $\times$  Tris-Buffered Saline (TBS) and 0.1% pancreatic elastase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were added to the samples and incubated at 4 °C overnight. The sulfated glycosaminoglycan (sGAG) content was measured with sGAG quantitative kit (Weislab Euro Diagnostica, Sweden) according to the manufacturer instruction.

## 2.8. Immunofluorescence

Unstained sectioned histology samples were deparaffinized and washed with PBS at room temperature for 20 min. Antigen retrieval was performed collagen type II using 2.5% Hyaluronidase (Sigma) in PBS at room temperature for 20 min. Nonspecific reactions were blocked by submerging samples in 10% bovine serum albumin (BSA) (Sigma–Aldrich) in PBS at room temperature for 20 min. The collagen type II were bound by primary polyclonal anti-rabbit collagen type II antibodies (LSL Co., Ltd., Tokyo, Japan) diluted with 1% BSA in PBS (1:200 dilution) at 4 °C overnight, and detected by Goat anti-Rabbit highly cross-absorbed secondary antibody Alexa Fluor 488 (ICN Biomedicals, Aurora, OH) diluted with 1% BSA in PBS (1:500 dilution) at room temperature for 30 min. Sections were mounted with ProLong Gold antifade reagent with DAPI (Life Technologies Corporation, Oregon USA).

## 2.9. Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analysis

To determine the messenger RNA levels of collagen type I, collagen type II, collagen type X, glial fibrillary acidic protein (*GFAP*), and *GAPDH* of the cells from the pHEMA and non-coated conditions, we collected the cells that had adhered to pHEMA coated and non-coated centrifuge tubes along with the cells in the suspension of the pHEMA and non-coated tubes. The cells were collected using Isogen (Nippon Gene, Tokyo, Japan), and the RNA was prepared with a chloroform extraction and isopropanol precipitation, according to manufacturer's instructions. The RNA concentration was measured with a spectrophotometer, and adjusted to obtain 1  $\mu\text{g}/\mu\text{L}$  of RNA. The RT-PCR sample was prepared according to guidelines provided by PrimeScript RT-PCR Kit (Takara Bio Inc., Shiga, Japan), and reverse transcribed using a thermal cycler to make complementary DNA. Primer F and Primer R (Invitrogen, Carlsbad, CA) (Table 1) for each target gene were added to SYBR Green FAST master mix (Invitrogen), distilled water, and the cDNA solution made in the previous step or standard solution. SYBR Green PCR amplification and real-time fluorescence detection were performed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). All reactions were run in quadruplicate. Copy numbers of target gene mRNA in each total RNA were calculated by reference to standard curves and were adjusted to the *GAPDH* copy number.

**Table 1**  
Sequence of primers used in real-time PCR.

Genes	Forward primer	Reverse primer
<i>GAPDH</i>	GAAGGTGAAGGTCGGAGTCA	GAAGATGGTGATGGGATTTC
<i>Col1A1</i>	ATTCCAGTTCGAGTATGGCG	CGACAGTGACGCTGTAGGTG
<i>Col1A1</i>	TTCAGCTATGGAGATGACAATC	AGACTCTAGAGTGACTGAG
<i>Col1A1</i>	AGGAATGCCTGTGTCTGCTT	ACAGGCCTACCCAACATGA
<i>GFAP</i>	CACCATGGAGAGGACGCA	CATCTCCACGGTCTTACCAC

## 2.10. Statistics

Cell proliferation, cell viability post-transfer, and colony formation unit, were expressed as mean values  $\pm$  standard deviations, with statistical significance defined as  $p < 0.05$  using paired t-tests.

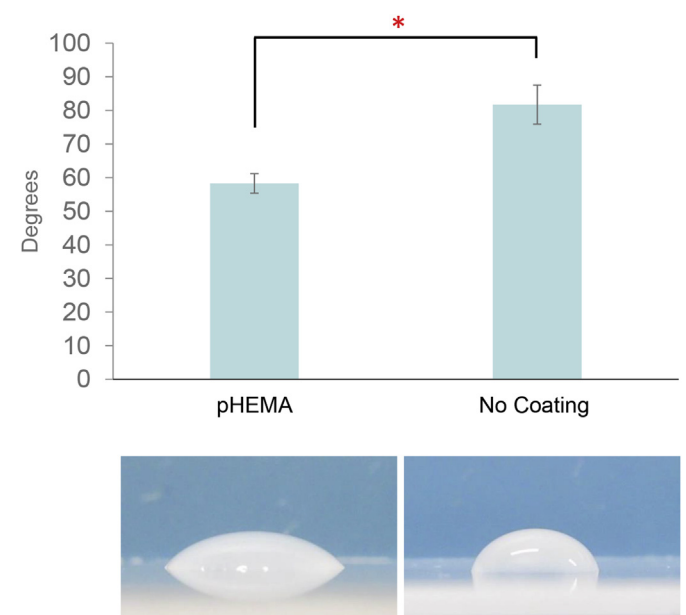
## 3. Results

### 3.1. Water contact angle

In order to confirm the intactness of the pHEMA coating layer on the polypropylene tubes used, we performed a water contact angle test. Hydrophilicity is signified by the dispersal of water, forming a spherically flat water droplet on the surface of the plate which in turn, forms a smaller contact angle. In contrast, if the surface of the plate is hydrophobic, the contact angle will be larger to reduce surface contact between the plate and the water molecules. Fifty  $\mu\text{L}$  of water was pipetted onto pHEMA-coated and non-coated plates. The acute angle formed between the plate and the corner of half-sphere of the water droplet was used to measure the water contact angle. The water angle formed on the pHEMA-coated plate was significantly smaller ( $p < 0.05$ ) than the non-coated tubes (Fig. 1), indicating the hydrophilicity of the pHEMA-coated surface, and hydrophobicity of the non-coated surface.

### 3.2. Cell recovery after multiple centrifugations

Once we confirmed that the pHEMA layer could be sufficiently coated onto the materials necessary for the experiment consisting

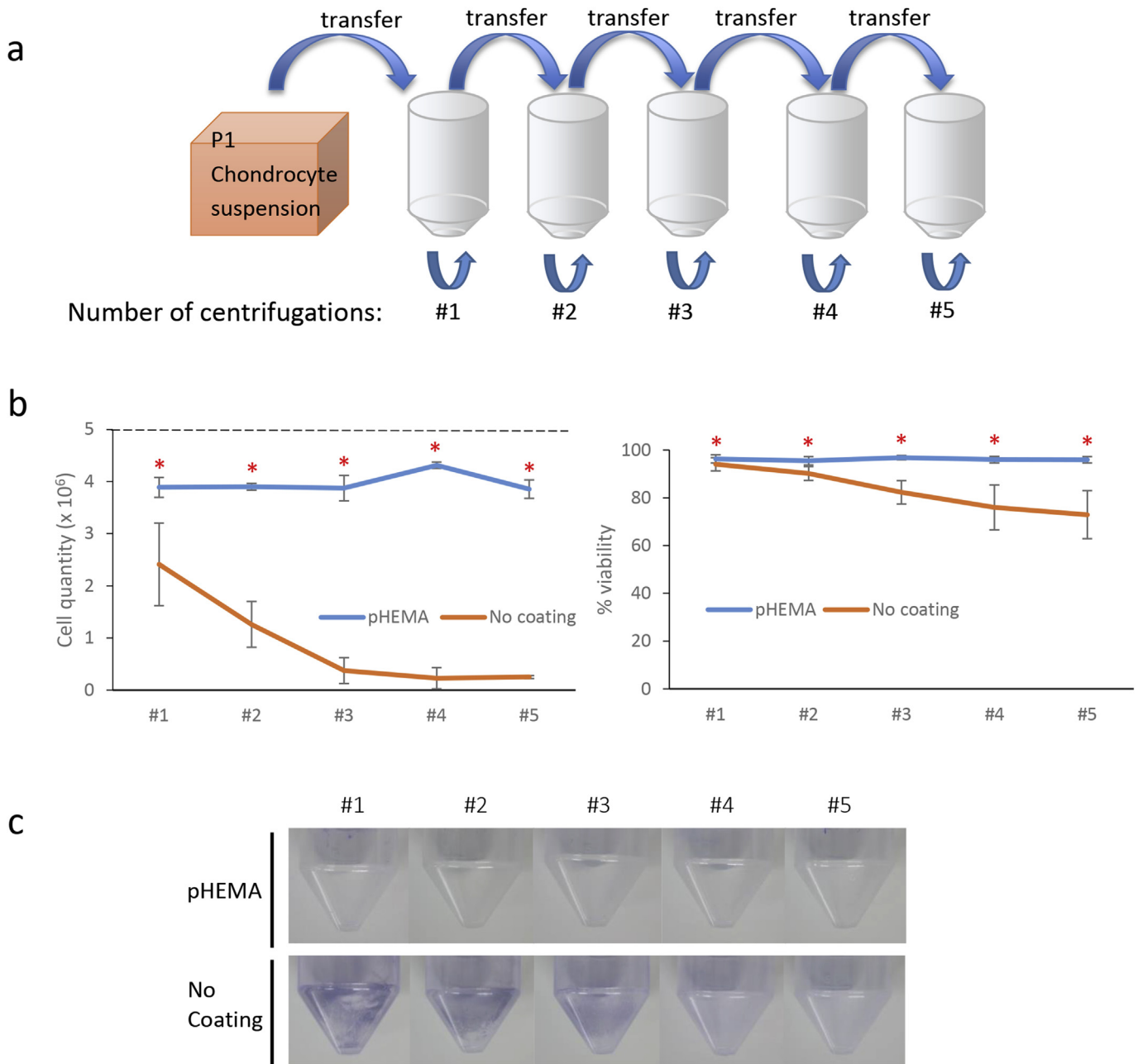


**Fig. 1.** Water contact angle difference between pHEMA and no coating tubes ( $n = 3$ ;  $p < 0.05$ ).

of serological pipettes, pipette tips, and 50 mL centrifuge tubes, the auricular chondrocytes that had undergone passage 1 cell expansion under the condition of monolayer culture were divided into two separate conditions. Chondrocyte suspensions containing  $5 \times 10^6$  cells/10 mL of control medium were pipetted using serological tubes into one of two groups (pHEMA coated or non-coated 50 mL centrifuge tubes), after which they were centrifuged, resuspended via thoroughly pipetting 5 times with the 25 mL pipette, and the number of cells in each suspension were counted using Nucleo Counter (Chemometec, Allerod, Denmark), with 4 additional repetitions (Fig. 2a). The cell suspension within the non-coated condition retained  $(3.2 \pm 1.8) \times 10^5$  cells by the end of the 5 transfers, while  $(3.9 \pm 0.16) \times 10^6$  cells were counted in the pHEMA coated condition suspension, indicating a statistically significant

( $p < 0.05$ ) 12-fold increase in cell quantity retention with the pHEMA coating (Fig. 2b, left graph). In addition, the cell viability decreased in the non-coated group, while it remained constant in the pHEMA-coated group during 5 centrifugations (Fig. 2b, right graph).

The presence of remnant cells from the 5 transfers was visually confirmed by staining the centrifuge tubes with Crystal Violet (Fig. 2c). The 5 pHEMA coated centrifuge tube had little to no purple stain, as compared to the 5 tubes that were not coated with pHEMA solution. In the tubes that were not coated with pHEMA, the first tube had the strongest stain. As the number of transfers increased, the intensity of the stain lessened gradually, indicating the decreasing quantity of cells within the cell suspension between transfers.



**Fig. 2.** a: Centrifuge 5× diagram, b: Number of cells (left) and viability (right) in the suspensions after centrifugation once to 5 times in pHEMA-coated and non-coated tubes ( $n = 3$ ; dotted line: initial count), c: Determination of cell adhesion to tubes with pHEMA coating on all materials, and no coating on materials.

### 3.3. Impact of centrifugation on cell adhesion

The number of cells counted after the first centrifugation set displayed a significant 2-fold greater retention in cell quantity for the suspension pipetted into the pHEMA coated tube compared to the suspension in the non-coated tube ( $p < 0.05$ ) (Fig. 3a). When the cell suspension was pipetted into the two respective tubes and incubated at room temperature for 5 min without centrifugation, the resulting differences between the cell quantity within the pHEMA and non-coated tubes were insignificant. The pHEMA and

non-coated 50 mL tubes used during this experiment were stained with Crystal Violet to visually demonstrate the presence of cells that had adhered to the tubes after centrifugation and resuspension. This was a positive confirmation of the valuable impact pHEMA has on cell quantity retention during centrifugation experimental procedures.

### 3.4. Chondrocyte proliferation and colony formation of cell suspension after centrifugation

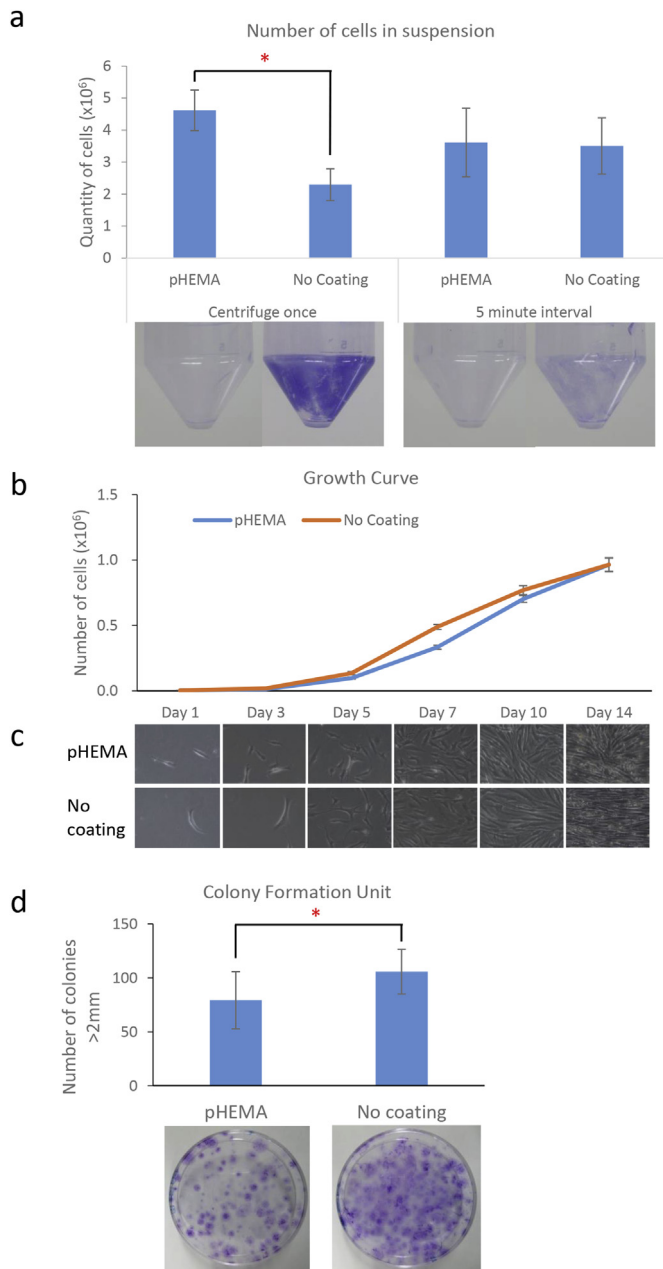
We compared the proliferation rate of the second passage monolayer culture of the auricular chondrocytes centrifuged once with the pHEMA and non-coated tubes. There was an insignificant difference in proliferation rates between the two groups (Fig. 3b), and associated gross microscopic view of the proliferating cells (Fig. 3c). Along with the observation of proliferation rate, the cells from the two conditions were also plated onto 100 mm Collagen Type I dishes at a density of 200 cells/10 mL of proliferation medium to quantify colony formation abilities. On day 14 of the monolayer culture of the cells extracted from the respective cell suspensions, the cells from the pHEMA coated group formed  $79.25 \pm 26.46$  colonies while the cells from the non-coated group formed  $105.75 \pm 20.69$  colonies ( $p < 0.05$ ; Fig. 3d).

### 3.5. Gene expression of suspended and adhered chondrocytes after centrifugation

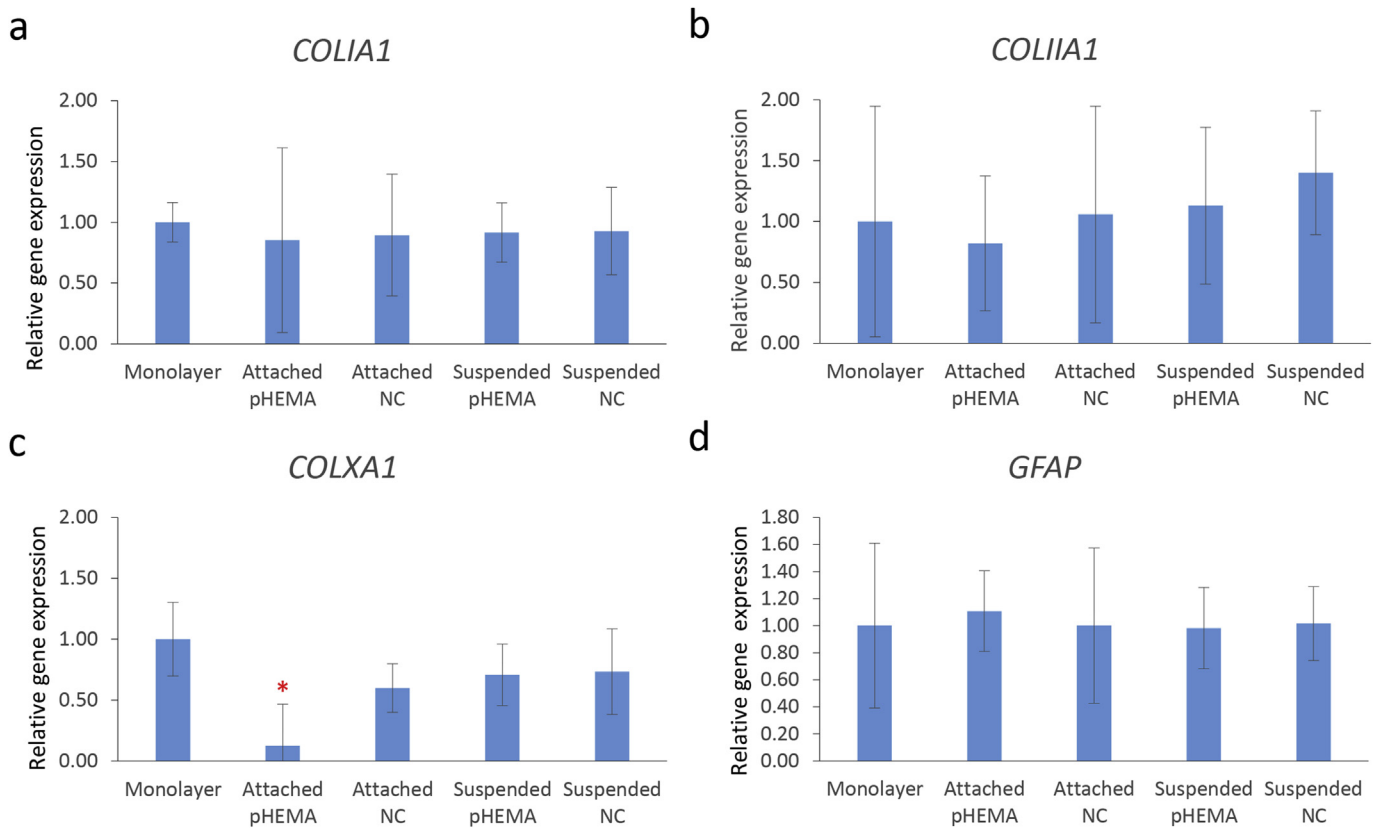
Having quantified the number of cells that had adhered to the centrifuge tubes after the centrifugation process under pHEMA and non-coated conditions, we then measured gene expression levels for *COL1A1*, *COL1A2*, *COL3A1*, and *GFAP* using RT-PCR. Auricular chondrocytes were monolayer cultured under passage 1 expansion in proliferation medium. The cells that had adhered to the pHEMA-coated and non-coated tubes after centrifugation, and cells in the cell suspension of the pHEMA and non-coated tube after centrifugation were harvested. All target genes were present at the same level with insignificant expression differences except for the expression of *COL3A1*, which was expressed in a statistically significant lower level in cells that had adhered to the pHEMA coated tubes after centrifugation ( $p < 0.05$ ) (Fig. 4).

### 3.6. Cartilage formation by chondrocytes after centrifugation

After having confirmed the cell quantity retention using pHEMA, we next examined the extracellular matrix production abilities of the two conditions. The auricular chondrocytes were cultured as passage 1, and they were harvested and separated into 2 groups. Chondrocyte suspensions of  $5 \times 10^6$  cells/10 mL of control medium were pipetted into a pHEMA coated and a non-coated 50 mL tube. They were then formed into 1% atelocollagen pellets and incubated for 3 weeks in differentiation medium, and subsequently transplanted into nude mice for 4 weeks (Fig. 5a). During the in vitro 3D culture, Toluidine Blue Staining and Hematoxylin & Eosin staining exhibited accumulation of cartilage matrices after 2 weeks (Fig. 5c,d). Immunofluorescence analyses were performed on the sectioned samples for collagen type II which appeared at higher intensity as the weeks progressed in both pHEMA and non-coated conditions (Fig. 5e) without any visual difference between the two conditions. sGAG content was measured resulting in statistically insignificant difference in cellular matrix production abilities between cell populations in the pHEMA and non-coated groups along with no significant changes in extracellular matrix content at the end of the three weeks compared to the first week (Fig. 5f). The pellets were transplanted into the nude mice for in vivo culture and harvested after 4 weeks. The pellets had a solid



**Fig. 3.** a: Number of cells within the cell suspension under pHEMA and no-coating tube conditions that were either centrifuged once ( $n = 12$ ) or incubated at room temperature for 5 min before being resuspended ( $n = 4$ ), b: Growth curve of cells from the centrifuge once condition seeded at a density of  $10^4$  cells/mL as passage 2 and observed for 14 days ( $n = 3$ ;  $p > 0.05$ ), c: Microscopic view of cells during proliferation, d: Comparison of number of colonies formed when 200 cells from each condition were plated onto dishes ( $n = 3$ ;  $p < 0.05$ ), and gross view of dishes of pHEMA condition (left) and non-coated condition (right).



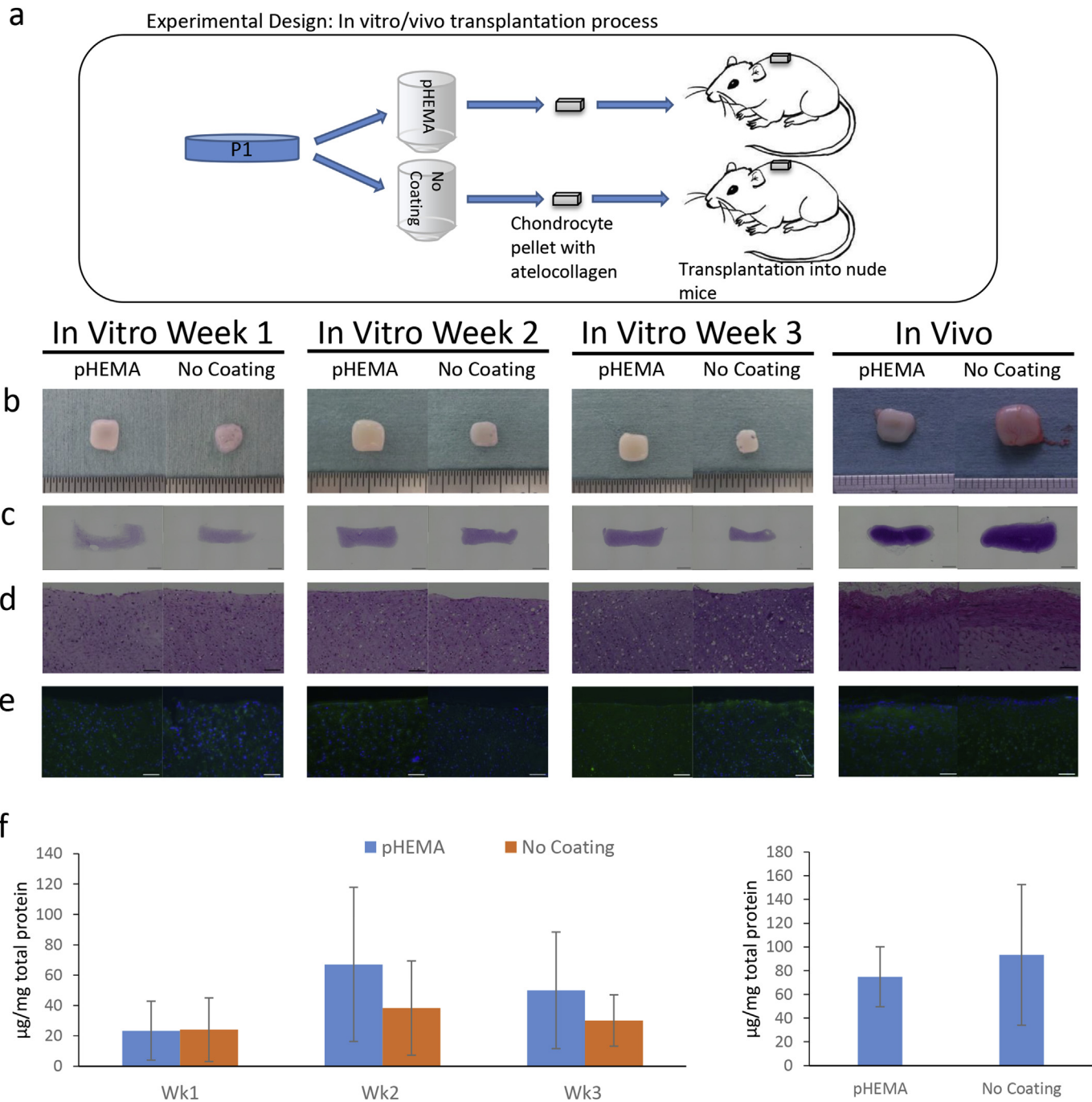
**Fig. 4.** a, b, c, d: qPCR gene expression level comparison for passage 1 cells, a. *COL1A1* expression levels, b. *COL1A1* expression levels, c. *COLXA1* expression levels, d. *GFAP* expression levels (n = 3; NC: no coating).

white color with a hardened texture (Fig. 5b). Histological observation revealed greater metachromasia in the in vivo 3D culture compared to the previous in vitro 3D culture (Fig. 5c,d). Further, immunofluorescence analysis showed similar levels of Collagen type II present in the matured pellets for both conditions, and no significant difference in sGAG content (Fig. 5e,f).

### 3.7. The effect of pHEMA application from earlier time points

When pHEMA was applied at passage 1, a significant reduction in cell loss throughout the process resulted, with little difference in matrix production. Thus we initiated the application of pHEMA coating on the materials used in earlier steps of the experiment (Fig. 6a). After obtaining the native auricular cartilage from microtia patients, the chondrocytes were isolated, separated from perichondrium and skin, digested with 0.3% collagenase, and filtered as was done in previous experiments. Instead of directly seeding it as a passage 0 monolayer culture, the cell suspension containing  $2 \times 10^6$  cells were pipetted into pHEMA coated and non-coated 50 mL tubes, thus starting the experiment from passage 0 original. These tubes were centrifuged and the quantity of cells remaining in the cell suspension was counted after resuspension. Compared to the cells in the non-coated tube, a statistically significant 1.87-fold increase in cells were counted for the cells within the pHEMA coated tube ( $p < 0.05$ ) (Fig. 6b). Throughout the following passages, we kept the pHEMA or non-coated conditions consistent to determine whether there would be any differences in colony formation, proliferative ability, or cartilage formation. The presence of more cells that had adhered onto the non-coated tubes was reconfirmed when the tubes used were stained with crystal violet (Fig. 6c). The respective suspensions were plated at a

concentration of  $2 \times 10^5$  cells/10 mL onto 5 plates of 100 mm collagen Type I dishes as passage 0 for proliferation observation for 7 days, and at a concentration of 200 cells/10 mL onto 3 plates of 100 mm collagen Type I dishes for colony formation unit analysis for 14 days. After 7 days of proliferation, the cells were collected using Trypsin – EDTA from dishes displaying confluency. The number of cells on the dishes from each condition was counted. The pHEMA condition was able to retain 2.3-fold times more cells comparatively to the non-coated condition ( $p < 0.05$ ) (Fig. 6d). After 14 days of incubation, the CFU dishes were stained with crystal violet resulting in similar number of colonies formed for both conditions ( $p > 0.05$ ) (Fig. 6f). The cell group that had been placed in the pHEMA tube after obtaining the auricular cartilage were then pipetted into new pHEMA coated 50 mL tubes. The same was done for the non-coated cell group into non-coated 50 mL tubes. Once pipetted, the cell suspensions in the two tubes were centrifuged and seeded as a monolayer culture at a density of  $2 \times 10^5$  cells in 10 mL of proliferation medium onto 10 plates of 100 mm collagen Type I dishes and cultured for 7 days, and another set of dishes for colony formation unit analysis were plated and cultured for 14 days. On day 7 and 14, the cells were collected and counted. The theoretical number of cells in the pHEMA coated group was  $2.1 \times 10^9$  cells, which was 2.2 times more than the theoretical cell yield of  $9.5 \times 10^8$  cells from the cell suspension in the non-coated condition after 2 rounds of monolayer culturing ( $p < 0.05$ ) (Fig. 6e). A statistically insignificant difference was observed between the two conditions in the number of colonies formed ( $p > 0.05$ ) (Fig. 6g). These cells that had undergone two centrifugations using pHEMA coated materials or non-coated materials were then formed into 1% atelocollagen pellets and cultured as passage 1. The histological analysis of the 3D pellets formed after 2



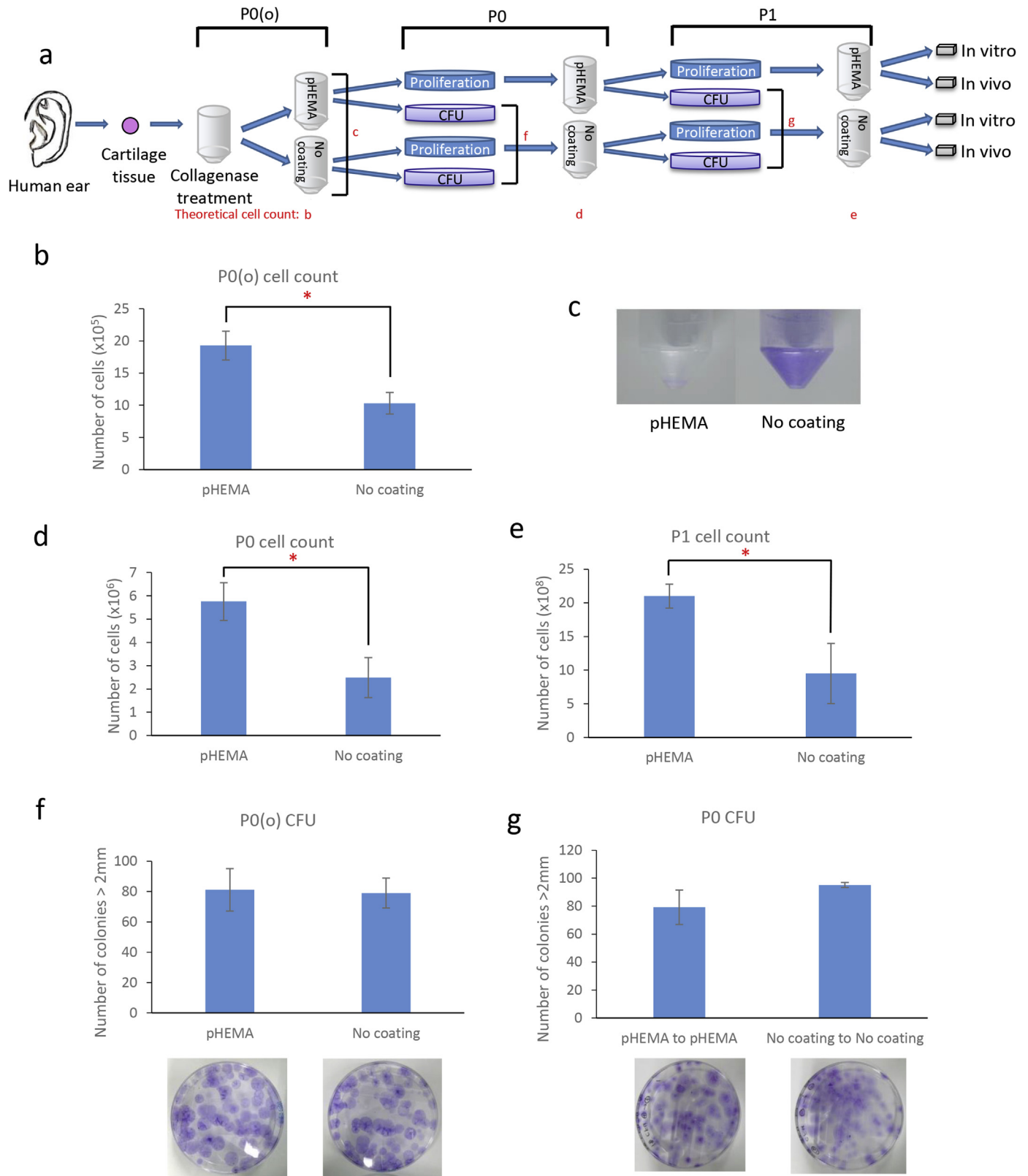
**Fig. 5.** P1 cells centrifuged in pHEMA and no coating conditions and formed into chondrocyte pellets incubated for 3 weeks in vitro and in vivo ( $n = 3$ ), a: Experimental design diagram of the monolayer and 3D culture of chondrocytic cells, b: Raw pellets (scale bar = 1 mm), c, d, e: Histological sections, c: Toluidine blue staining (scale bar = 100  $\mu\text{m}$ ), d: Hematoxylin & Eosin staining (scale bar = 50  $\mu\text{m}$ ), e: COL2 immunofluorescence staining (scale bar = 100  $\mu\text{m}$ ), f: sGAG contents ( $n = 3$ ; values adjusted by total protein content) Wk 1:  $p > 0.05$ , Wk 2:  $p > 0.05$ , Wk 3:  $p > 0.05$ , in vivo:  $p > 0.05$ .

passages under pHEMA- and non-coated conditions (Fig. 7) had consistent results as the 3D pellets formed after only 1 passage (Fig. 5). The 3D pellets from the pHEMA-coated condition had no apparent difference in size nor matrix production abilities ( $p > 0.05$ ) within both in vitro and in vivo cultures.

#### 4. Discussion

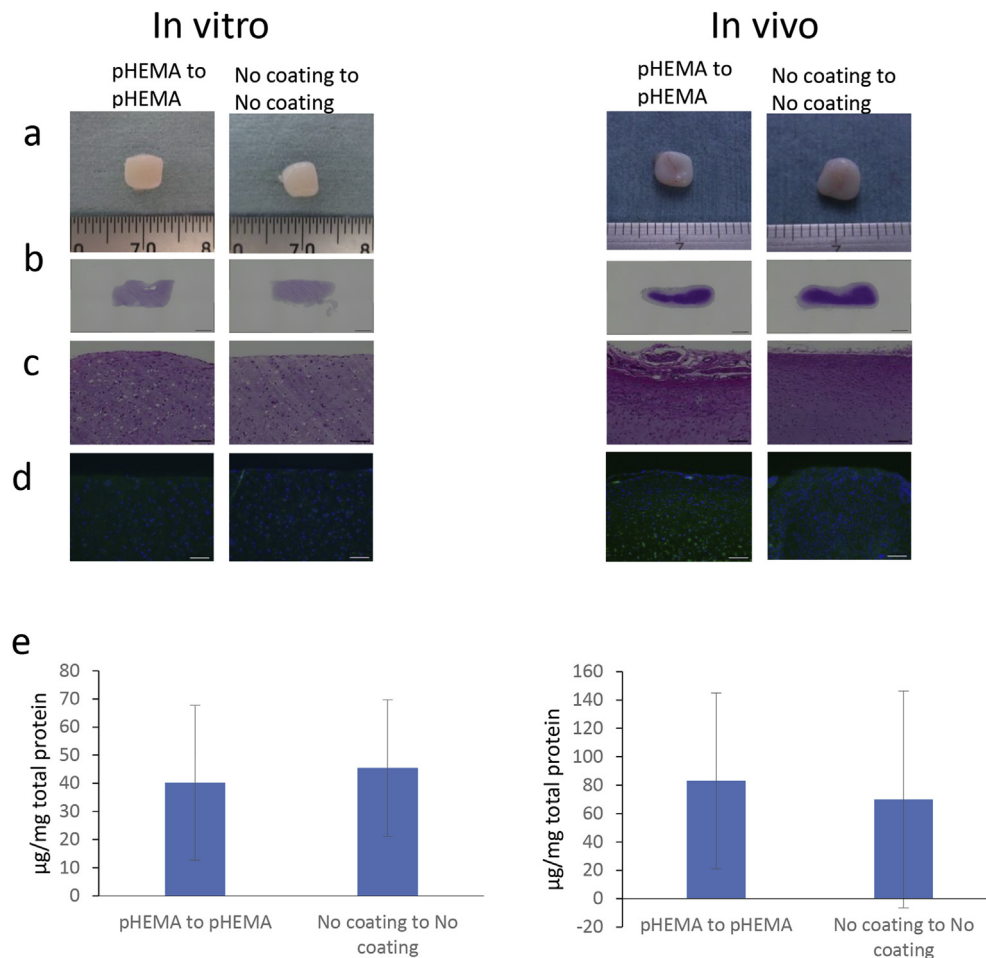
In this study, pHEMA was applied to materials used during cell culturing procedures to determine the degree of antifouling

character it possess and to find possible benefits to the chondrocyte population harvested. Among antifoulants, in addition to pHEMA, many other polymers have been used in previous research for the purpose of inhibiting cell adhesion. They have included polyethylene glycol (PEG), 2-methacryloyloxyethyl phosphorylcholine (MPC), sulfobetaine methacrylate (SBMA), and carboxybetaine methacrylate (CBMA) [25–27]. Antifouling agents are primarily divided into two categories that define resistance to cell adhesion: polyhydrophilic (PEG, pHEMA) or polyzwitterionic character (phosphobetaine, sulfobetaine, and carboxybetaines). Zwitterionic



**Fig. 6.** Native auricular cartilage P0(o) a: Experimental Design, b: Number of cells within the cell suspension of the pHEMA-coated and non-coated tubes after the initial centrifugation and resuspension of P0(o) chondrocytes (n = 3), c: pHEMA and non-coated tubes after centrifugation stained with Crystal Violet for cell adhesion, d: Number of cells in suspension from P0 cell harvest (n = 3), e: Cell numbers in the suspension after P1 cells were harvested and divided into pHEMA and non-coated group (n = 3), f: Number of colonies formed and gross view of dishes with cells seeded as passage 0 at a density of 200 cells/10 mL for colony formation analysis in pHEMA-coated conditions (left) and non-coated conditions (right) (n = 3), g: Number of colonies formed after the native auricular cartilage was cultured for 7 days and seeded onto dishes for colony formation analysis with pHEMA and non-coated condition divisions (n = 3).





**Fig. 7.** In vitro (left) and In vivo (right) histological analyses of 3D pellets of pHEMA- and non-coated conditions ( $n = 3$ ), a: Raw pellets (scale bar = 1 mm), b: Toluidine staining (scale bar = 100  $\mu\text{m}$ ), c: Hematoxylin & Eosin staining (scale bar = 50  $\mu\text{m}$ ), d: COL2 immunofluorescence (scale bar = 100  $\mu\text{m}$ ), e: sGAG content of pellets (values adjusted by total protein content;  $p > 0.05$  for both in vitro and in vivo cultures).

organic molecules are characterized as having both cationic and anionic properties, enabling greater electrostatic bonds to form with water molecules in their surroundings compared to the hydrogen bonds of polyhydrophilic organic molecules [28]. However, disadvantages to these polyzwitterionic polymers have restricted its application, including complex synthesizing procedures [29], low packing density for some sulfobetaines [30], and limited commercial availability [31].

Recently, attention has been given to PEG oligomers, which have been widely used to reduce non-specific protein absorption [31,32]. Yet, in certain instances, generation of reactive oxygen species has contributed to apoptotic effects, if not supplemented with antioxidants as well as limited long term usage [31–35]. Chemical structure previously discussed, and the application technique of the antifoulant synergistically contributes to the functionality of the bioreagent. Methods such as graphed-on surface, graphed-from surface, self-assembled monolayer (SAM), and spin-/dip-coating application techniques are potential candidates. Methods such as grafting-to and SAM approaches require specific surface chemistry for polymer chains to attach, limiting the applicability of substrates to surfaces [33]. Despite extensive investigation of similar hydrophilic polymers and application techniques, very few dip-coating applications have been applied and none have been extensively characterized.

As a neutrally charged hydrophilic chemical with a flexible poly-chain [13,26], pHEMA has three characteristics common to all antifouling agents include hydrophilicity, electrical neutrality, and the ability to form hydrogen bonds [26]. The poly-hydrophilic chemical structure of a compound was confirmed with the increased contact to surface and shallow water contact angle (Fig. 1). The hydrophilicity contributes to the ability to create hydrogen bonds with water, forming a hydration layer that in turn provides antifouling effects on cells and other substrates coming in contact with the poly-hydrophilic coating [36]. By limiting energy exchanges between charges on cell surfaces and material surfaces, surface hydration allows for thermodynamic stability through achieving favorable entropy. The term “interfacial energy matching” was used to describe this antifouling mechanism [25]. The overall good properties of pHEMA including no proven cytotoxic effects, strong antifouling characteristic, and affordability make it a compatible resource for standard lab procedures with far-reaching effects.

In applying pHEMA to the centrifugation tubes during 5 rounds of repeated centrifugations and resuspension procedures, the number of cells collected from the suspension was 12 times greater in the cell suspension with the pHEMA coated condition compared to the cells that had underwent the repetitions without pHEMA coated centrifuge tubes. As the number of centrifugation

repetitions increased, the percentage of viable cells within the cell suspension decreased significantly for the cells in the non-coated tubes. This trend was not observed within the sample of cells in the pHEMA coated condition. Potential mechanical stress elicited from centrifugation and resuspension can be alleviated by the hydration layer separating the tube and the cell suspension. The experiment was carried out by another experimenter to reconfirm the significant 12-fold decrease in cell count. The finding was consistent with another study that attempted to quantify the cell losses during centrifugation procedures. The study found triple the number of losses in cell yield when normal tubes were used as compared to when pHEMA-coated tubes were used during the collection of corneal endothelial cells from corneal endothelial tissues [21].

In our study, we were able to confirm the major role centrifugation plays on cell loss. Cells centrifuged in 50 mL centrifuge tubes for 5 min lost half of the P1 cells within the cell suspension due to the adherence of cells to the tube after centrifugation and resuspension. In comparison, the cells that were held in 50 mL centrifuge tubes for the same 5-min duration without centrifugation displayed minimal adhesion, demonstrating the strong adhesive impact centrifugation has on suspensions. Gene expression levels for chondrocytes that had adhered to the side of the tubes and those that had been dislodged from the tubes through resuspension were measured. The genes measured through RT-PCR consisted of *COL1A1*, *COL1A1*, *COLXA1*, and *GFAP*—cellular markers for fibroblasts, chondrocytes, hypertrophic chondrocytes, and auricular chondrocytes [24] respectively. The chondrocytes that had adhered to the sides of the tubes and chondrocytes that had been resuspended for both pHEMA and non-coated conditions displayed similar fibroblastic, chondrocytic, and auricular chondrocytic marker levels. Yet, the hypertrophic chondrocyte gene expression levels were dramatically decreased for the chondrocytes that had remained attached to the side of the pHEMA coated tubes post-centrifugation and resuspension.

The chondrocytes obtained from the cell suspension in both conditions presented no difference in proliferation abilities or matrix production. More colonies were formed in the non-coated condition suggesting the positive impact that pHEMA may have on the density of chondrocyte stem/progenitor cells. Thus, further attempts to distinguish the effects of pHEMA culminated in its application at earlier stages of the cell culturing procedure. In performing the P0(o) experiment, the cellular matrix production and proliferation capabilities of the pHEMA- and non-coated condition chondrocyte pellets remained indistinguishable as expected. However, the colony formation capabilities differed from that of pHEMA utilization at later stages of the cell culturing protocol. The discrepancy in colony formation abilities was explained by focusing on protein markers displayed by the chondrocytes in both pHEMA- and non-coated conditions. Collagen type X is a chondrocyte maturation marker. The decreased gene expression of *COLXA1* for the attached cells of the pHEMA-coated condition indicates the cell population affected by the application of pHEMA is that of matured chondrocytes. This phenomenon provides a possible explanation for the difference in colony formation ability within pHEMA and non-coated conditions. With a greater number of mature chondrocytes in the cell suspension of the pHEMA condition, the density of colony forming cells (Cartilage-derived progenitor cells—CSPCs) [37,38] that have stem cell-like abilities within the cell suspension could be the smaller. They are seeded onto the 100 mm dishes for CFU analysis at the same cell concentration as the non-coated condition, resulting in less colony formation observed. A discrepancy was observed for the colony formation ability of cells when pHEMA was introduced earlier in the P0(o) experiment. The pHEMA coated condition cells that were inoculated for colony

formation analysis immediately at passage 0 (original) and those that were inoculated a week later using the same chondrocyte population both showed no evidence of pHEMA effect. With the increased expansion of cells through passaging, more mature chondrocytes can be harvested with the application of pHEMA, and in effect, decreasing the colony formation abilities of the cell population. Although the proliferation rates among cells in the pHEMA- and non-coated condition remained constant, the application of pHEMA increased the total theoretical number of cells after 2 expansions to 2.2 times more than standard procedures without the application. Additionally, no evidence was found for significant change in size and cell matrix production integrity of 3D culture pellets, thus providing a safe chondrocyte centrifugation method.

## 5. Conclusion

This study provides an innovative method for improving cell yield during passages. The number of chondrocytes harvested was significantly increased when a pHEMA coating was applied to materials. This was most evident when cells were transferred into pHEMA and non-coated centrifuge tubes and centrifuged, resulting in a difference in degree of cell adhesion to the side and bottom of the tube due to centripetal acceleration. Regenerative cartilage constructs formed from auricular chondrocytes of the two conditions yielded similar amounts of extracellular matrix production, indicating no degradation of cellular quality with the use of pHEMA.

Further studies are needed to analyze properties within the cell population treated with pHEMA coating materials, and evaluate the impact on stem/progenitor cells within a chondrocyte population. However, the pHEMA coating is expected to increase the amount of cell harvests and maintain the quality of cell sources essential for regenerative medicine.

## Acknowledgements

This work was supported by Grants-in-Aid for Regenerative Medicine Development Support Program (National Institutes of Biomedical Innovation, Health and Nutrition). We would like to thank Mr. Tomoaki Sakamoto, Mrs. Dan Liu, and Mrs. Akiko Takakura for their technical assistance as well as Dr. Seiichi Yokoo and Mrs. Hiroko Komura, for introducing the topic of pHEMA to us without which our curiosity may not have arisen.

## References

- [1] Fukuda O. The microtic ear: survey of 180 cases in 10 years. *Plast Reconstr Surg* 1974;53:458–63.
- [2] Yamauchi M, Yotsuyanagi T, Ikeda K, Yoshikawa M, Urushidate S, Mikami M, et al. Clinical and genetic analysis of microtia in Japan. *J Plast Surg Hand Surg* 2012;46:330–4. <http://dx.doi.org/10.3109/2000656X.2012.700018>.
- [3] Hoshi K, Fujihara Y, Saijo H, Asawa Y, Nishizawa S, Kanazawa S, et al. Implant-type tissue-engineered cartilage for secondary correction of cleft lip-nose patients: an exploratory first-in-human trial. *J Clin Trials* 2017 Jun;7(3). <http://dx.doi.org/10.4172/2167-0870.1000315>.
- [4] Otani Y, Komura M, Komura H, Ishimaru T, Konishi K, Komuro H, et al. Optimal amount of basic fibroblast growth factor in gelatin sponges incorporating beta-tricalcium phosphate with chondrocytes. *Tissue Eng A* 2015 Nov;21:627–36. <http://dx.doi.org/10.1089/ten.tea.2013.0655>.
- [5] Vacanti CA, Vacanti JP. Bone and cartilage reconstruction with tissue engineering approaches. *Otolaryngol Clin North Am* 1994;27:263–76.
- [6] Tanaka Y, Yamaoka H, Nishizawa S, Nagata S, Ogasawara T, Asawa Y, et al. The optimization of porous polymeric scaffolds for chondrocyte/atelocollagen based tissue-engineered cartilage. *Biomaterials* 2010 Jun;31(15):4506–16. <http://dx.doi.org/10.1016/j.biomaterials.2010.02.028>.
- [7] Shieh SJ, Terada S, Vacanti JP. Tissue engineering auricular reconstruction: in vitro and in vivo studies. *Biomaterials* 2004 Apr;25(9):1545–57. [http://dx.doi.org/10.1016/S0142-9612\(03\)00501-5](http://dx.doi.org/10.1016/S0142-9612(03)00501-5).
- [8] Yamaoka H, Tanaka Y, Nishizawa S, Asawa Y, Takato T, Hoshi K. The application of atelocollagen gel in combination with porous scaffolds for cartilage

- tissue engineering and its suitable conditions. *J Biomed Mater Res A* 2010;93(123).
- [9] Hoshi K, Fujihara Y, Asawa Y, Nishizawa S, Kanazawa S, Sakamoto T, et al. Recent trends in cartilage regenerative medicine and its application to oral and maxillofacial surgery. *Oral Sci Int* 2012 May;10:15–9. [http://dx.doi.org/10.1016/S1348-8643\(12\)00049-3](http://dx.doi.org/10.1016/S1348-8643(12)00049-3).
- [10] Yonenaga K, Nishizawa S, Fujihara Y, Asawa Y, Kanazawa S, Nagata S, et al. The optimal conditions of chondrocyte isolation and its seeding in the preparation for cartilage tissue engineering. *Tissue Eng Part C Methods* 2010 Dec;16(6):1461–9. <http://dx.doi.org/10.1089/ten.TEC.2009.0597>.
- [11] Yonenaga K, Nishizawa S, Fujihara Y, Asawa Y, Kanazawa S, Nagata S, et al. Application of floating cells for improved harvest in human chondrocyte culture. *Biomed Res* 2012;33(5):281–9. <http://dx.doi.org/10.2220/biomedres.33.281>.
- [12] Delahaye M, Lawrence K, Ward SJ, Hoare M. An ultra scale-down analysis of the recovery by dead-end centrifugation of human cells for therapy. *Biotechnol Bioeng* 2015 May;112(5):997–1011. <http://dx.doi.org/10.1002/bit.25519>.
- [13] Kiremitci M, Pesmen A, Pulat M, Gurhan I. Relationship of surface characteristics to cellular attachment in PU and PHEMA. *J Biomater Appl* 1993 Jan;7(3):250–64. <http://dx.doi.org/10.1177/088532829300700304>.
- [14] Hoffman AS. Hydrogels for biomedical applications. *Adv Drug Deliv Rev* 2012 Dec;64:18–23. <http://dx.doi.org/10.1016/j.addr.2012.09.010>.
- [15] Lopez GP, Ratner BD, Rapoza RJ, Horbett TA. Plasma deposition of ultrathin films of poly(2-hydroxyethyl methacrylate): surface analysis and protein absorption measurements. *Macromolecules* 1993 Jun;26(13):3247–53. <http://dx.doi.org/10.1021/ma00065a001>.
- [16] Li CC, Chauhan A. Ocular transport model for ophthalmic delivery of timolol through pHEMA contact lenses. *J Drug Deliv Sci Technol* 2007;17(1):69–79. [http://dx.doi.org/10.1016/S1773-2247\(07\)50010-9](http://dx.doi.org/10.1016/S1773-2247(07)50010-9).
- [17] Faccia PA, Pardini FM, Amalvy JL. Evaluation of pH-sensitive poly(2-hydroxyethyl methacrylate-co-2-(diisopropylamino)ethyl methacrylate) copolymers as drug delivery systems for potential applications in ophthalmic therapies/ocular delivery of drugs. *Express Polym Lett* 2015 Jan;9(6):554–66. <http://dx.doi.org/10.3144/expresspolymlett.2015.52>.
- [18] Bayramoglu G, Arica MY. A novel pH sensitive porous membrane carrier for various biomedical applications based on pHEMA/chitosan: preparation and its drug release characteristics. *Macromol Symp* 2003 Oct;203(1):213–8. <http://dx.doi.org/10.1002/masy.200351321>.
- [19] Ahmed EM. Hydrogel: preparation, characterization, and applications: a review. *J Adv Res* 2015 Mar;6(2):105–21. <http://dx.doi.org/10.1016/j.jare.2013.07.006>.
- [20] Fujimoto M, Ohte S, Shin M, Yoneyama K, Osawa K, Miyamoto A, et al. Establishment of a novel model of chondrogenesis using murine embryonic stem cells carrying fibrodysplasia ossificans progressiva-associated mutant ALK2. *Biochem Biophys Res Commun* 2014;455:347–52. <http://dx.doi.org/10.1016/j.bbrc.2014.11.012>.
- [21] Yokoo S, Yamagami S, Shimada T, Usui T, Sato T, Amano S, et al. A novel isolation technique of progenitor cells in human corneal epithelium using non-tissue culture dishes. *Stem Cells* 2008 Apr;26(7):1743–8. <http://dx.doi.org/10.1634/stemcells.2007-0866>.
- [22] Zoro BJ, Owen S, Drake RA, Mason C, Hoare M. Regenerative medicine bio-processing: concentration and behavior of adherent cell suspensions and pastes. *Biotechnol Bioeng* 2009 Aug;103(6):1236–47. <http://dx.doi.org/10.1002/bit.22356>.
- [23] Oster G, Pollister AW. Cells and tissues: physical techniques in biological research, vol. 3. New York: Academic Press; 1956. p. 316–25 [Print].
- [24] Kanazawa S, Nishizawa S, Takato T, Hoshi K. Biological roles of glial fibrillary acidic protein as a biomarker in cartilage regenerative medicine. *J Cell Physiol* 2017 Jan;9999:1–12. <http://dx.doi.org/10.1002/jcp.25771>.
- [25] Gong P, Grainger DW. Nonfouling surfaces A review of principles and applications for microarray capture assay designs. *Methods Mol Biol* 2007 Feb;381:59–92. [http://dx.doi.org/10.1007/978-1-59745-303-5\\_3](http://dx.doi.org/10.1007/978-1-59745-303-5_3).
- [26] Chen S, Li L, Zhao C, Zheng J. Surface hydration: principles and applications toward low-fouling/nonfouling biomaterials. *Polymer* 2010 Oct;51(23):5283–93. <http://dx.doi.org/10.1016/j.polymer.2010.08.022>.
- [27] Liu G, Iwata K, Ogasawara T, Watanabe J, Fukazawa K, Ishihara K, et al. Selection of highly osteogenic and chondrogenic cells from bone marrow stromal cells in biocompatible polymer-coated plates. *J Biomed Mater Res* 2010 Mar;92A(4):1273–82. <http://dx.doi.org/10.1002/jbm.a.32460>.
- [28] Jiang S, Cao Z. Ultralow-fouling, functionalizable, and hydrolyzable zwitterionic materials and their derivatives for biological application. *Adv Mater* 2010 Mar;22(9):920–32. <http://dx.doi.org/10.1002/adma.200901407>.
- [29] Ueda T, Oshida H, Kurita K, Ishihara K, Nakabayashi N. Preparation of 2-methacryloyloxyethyl phosphorylcholine copolymers with alkyl methacrylates and their blood compatibility. *Polym J* 1992 Apr;24(11):1259–69.
- [30] Zhang Z, Vaisocherova H, Cheng G, Yang W, Xue H, Jiang S. Nonfouling behavior of polycarboxybetaine-grafted surfaces: structural and environmental effects. *Biomacromolecules* 2008 Sept;9(10):2686–92. <http://dx.doi.org/10.1021/bm800407r>.
- [31] Damodaran VB, Murthy NS. Bio-inspired strategies for designing antifouling biomaterials. *Biomaterials Res* 2016 Jun;20(18). <http://dx.doi.org/10.1186/s40824-016-0064-4>.
- [32] Herold DA, Keil K, Bruns DE. Oxidation of polyethylene glycols by alcohol dehydrogenase. *Biochem Pharmacol* 1989 Jan;38(1):73–6. [http://dx.doi.org/10.1016/0006-2952\(89\)90151-2](http://dx.doi.org/10.1016/0006-2952(89)90151-2).
- [33] Kim M, Schmitt S, Choi JW, Kruty JD, Gopalan P. From self-assembled monolayers to coatings: advances in the synthesis and nanobio applications of polymer brushes. *Polymers* 2015;7(7):1346–78. <http://dx.doi.org/10.3390/polym7071346>.
- [34] Liu G, Li Y, Yang L, Wei Y, Wang X, Wang Z, et al. Cytotoxicity study of polyethylene glycol derivatives. *RSC Adv* 2017 Mar;7:18252–9. <http://dx.doi.org/10.1039/C7RA00861A>.
- [35] Sung H, Luk A, Murthy NS, Liu E, Jois M, Joy A, et al. Poly(ethylene glycol) as a sensitive regulator of cell survival fate on polymeric biomaterials: the interplay of cell adhesion and pro-oxidant signaling mechanisms. *Soft Matter* 2010 Aug;6(20):5196–205. <http://dx.doi.org/10.1039/C0SM00172D>.
- [36] Zhao C, Lil L, Wang Q, Yu Q, Zheng J. Effect of film thickness on the antifouling performances of poly(hydroxy-functional methacrylates) grafted surfaces. *Langmuir* 2011 Apr;27(8):4906–13. <http://dx.doi.org/10.1021/la200061h>.
- [37] Jiang Y, Tuan RS. Origin and Function of cartilage stem/progenitor cells in osteoarthritis. *Nat Rev Rheumatol* 2015 Apr;11(4):206–12. <http://dx.doi.org/10.1038/nrrheum.2014.200>.
- [38] Jiang Y, Cai Y, Zhang W, Yin Z, Hu C, Tong T, et al. Human cartilage-derived progenitor cells from committed chondrocytes for efficient cartilage repair and regeneration. *Stem Cells Transl Med* 2016 Jun;5(6):733–44. <http://dx.doi.org/10.5966/sctm.2015-0192>.