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A New Injectable Biphasic Hydrogel Based on Partially Hydrolyzed Polyacrylamide and Nano Hydroxyapatite, as Scaffold for Osteochondral Regeneration

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Supporting Information

I) Sample preparation and cell encapsulation protocol

II) Cytotoxicity assessments

III) Functional assessment

IV) *Real Time-PCR analysis*

V) Cell attachment

I) Sample preparation and cell encapsulation protocol

The preheated solutions were incubated into an ice bath to decline the temperature before cell encapsulation, and then the cell suspended samples were allowed to be completely gelled in a 5% CO_2 incubator at 37 °C. Our criteria to optimize hydogel composition were to obtain an injectable solution with a short gelation time under physiological condition and to achieve a biocompatible scaffold with an acceptable mechanical strength.

To optimize formulation, the gelation time of HPAM water solutions, containing 0.2 wt. % Cr (III), with polymer concentration of 0.5, 1, 2, 4 and 5 wt. % was first estimated by test tube inverting method, and the time at which no fluidity was observed was recorded as gelation time. Then, the Optimum HPAM water solution was mixed with various Cr (III) concentrations, including 0.06, 0.1, 0.2, 0.4 wt. % so that the optimized concentration of Cr (III) was also obtained.As Cr (III) in high levels was toxic to the encapsulated cells, biological assays were also examined along with this step.

In order to design a biphasic structure, besides the optimum hydrogel solution obtained from the visual method, similar solution containing 50 wt. % nHAp was also prepared. The concept of nHAp addition is to improve the integration of the hydrogel with the subchondral bone. After preheating and cooling, MSCs were encapsulated into each individual solution, and then the two hydrogel precursors were put in contact to each other to be crosslinked at interface in the form of two phases at 37 °C.

II) Cytotoxicity assessments

Toxicity of Cr(III) solutions was performed on MSCs using (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride (neutral red, Sigma) assay. After culturing 1×10^4 cells/well in a 96-well platefor 24 hours, the culture medium was replaced with 100 µl of various concentrations of filter sterilized Cr (III) solutions, including 0, 0.06, 0.1,0.125, 0.2, 0.3, 0.4, 0.5, 1 and 2 wt. %prepared in DMEM supplemented 10% FBS. The solutions were then aspirated from each well, and the neutral staining was done according to the agreed protocol published before. To quantify cell viability, the optical density (OD) of the colored solutions

wasmeasuredat 540 nm using ELISA reader (STAT FAX 2100, USA), and the results were normalized to the controlsample.

The viability study on hydrogels encapsulated MSCs was performed with agar diffusion and a live-dead assay. Agar diffusion was applied according to ISO 10993 Part 5. 2×10^4 cells/well was cultured in 24-well plate for 24 hours. The culture medium was then replaced with 300 µl of 1.5 % nutrient agar (Sigma, USA) in PBS.Subsequently, each cylindrical sample with dimension of (height= 10mm, diameter= 5mm) were overlaid on solidified agar gel following the addition of culture medium and incubation at 37 °C for 7 days. As a positive control, cells were also cultured in the absence of HPAM hydrogels. Finally, to quantify cell viability the cells were stained with neutral red. A stock of 670 µM Acridine orange (AO) and 750 µM Propidium iodide (PI) was also utilized for visualization of live and dead cells encapsulated in hydrogels [1]. The hydrogels containing 10^6 cells/ml were stained after 7 days in vitro culturing and observed by a fluorescence microscope (Zeiss, Germany) to distinguish between the viable (green) and dead (red) cells.

Figure S-II.1A indicates no serious cytotoxicity of the solutions containing lower than 0.2 wt. % Cr (III), while in the Cr (III) concentrations higher than 0.2 wt. %, local necrosis with significant toxicity was observed. Figures S-II.1B and C demonstrate the results of agar diffusion test and AO/PI staining of the cells exposed to the hydrogels, which were in agreement with the Cr (III) cytocompability analysis obtained above; suggesting no serious cytotoxic effects on cells exposed to the HPAM hydrogels with Cr (III) concentration up to 0.2 wt. %. It has been reported that as Cr (III) lacked the ability to permeate cell membrane, it is nontoxic at low concentrations and the high toxicity of chromium is contributed to the high permeation of chromium to cell membrane and causing DNA damage [6]. As a result, to have a hydrogel with both desirable biocompatibility and mechanical strength, 0.2 wt. % Cr (III) solution was considered as a threshold value to efficiently crosslink HPAM solutions.In

addition, no significant differences were observed between the cell viability of the HPAM3 and HPAM6 composite hydrogels, indicating the biocompatibility of the synthesized nHAP.

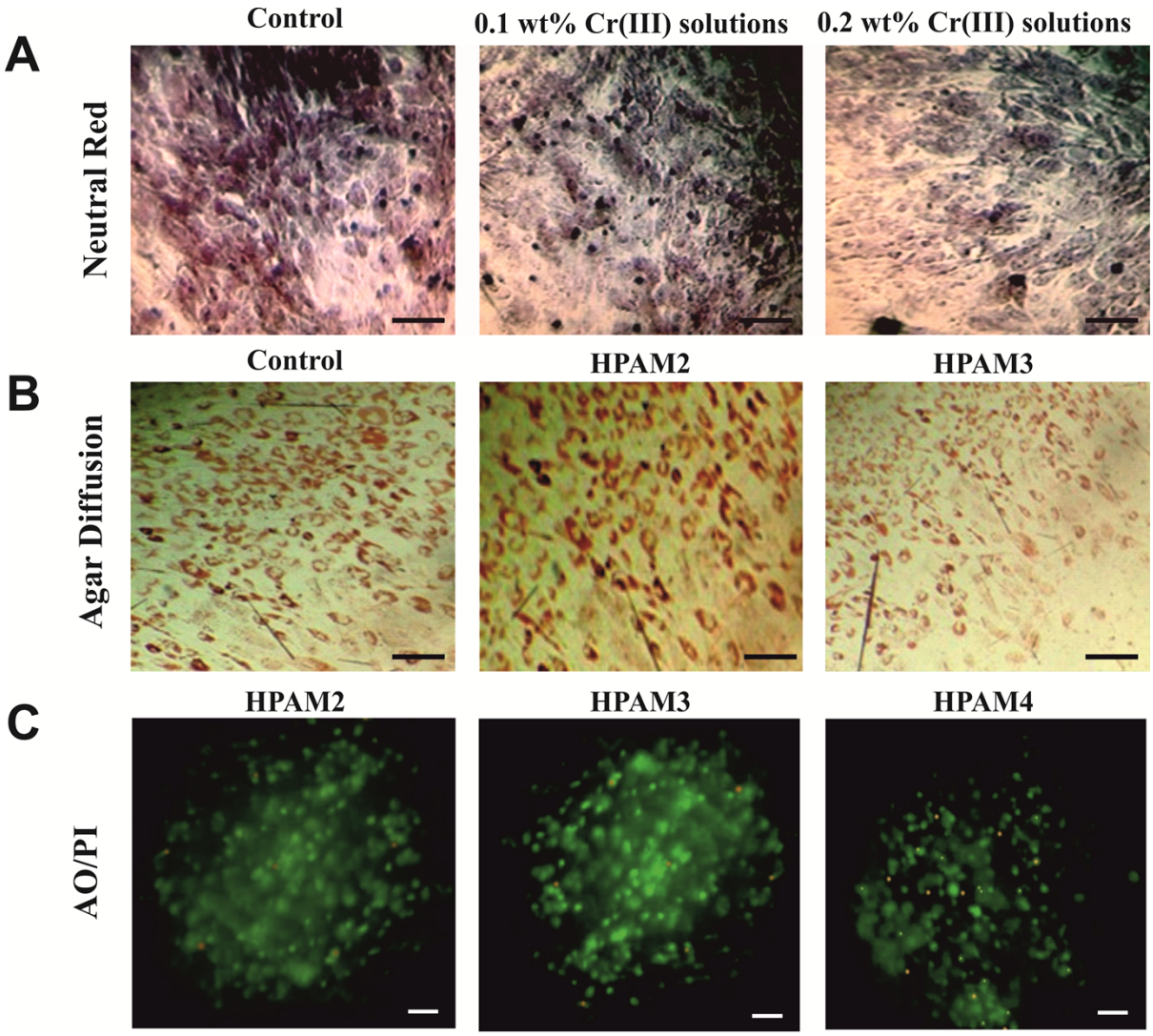

Fig. S-II.1 Neutral red staining of MSCs after A) 1 day exposure to Cr (III) solutions and B) 7 days exposure to HPAM hydrogels in agar diffusion test. C) Live-dead fluorescent staining ofMSCs incorporated into the various HPAM hydrogels with Initial cell density of 10^6 cells/ml after 7 days in culture (Scale bar represent 100 µm).

III. Functional assessment

III.1.Chondrogenic evaluation. In order to determine proteoglycan expression of the HPAM3 encapsulated MSCs (10^6 cells/ml),the hydrogel/cell constructs were stained with

Alcian blue (Sigma, USA) and Safranin O (Sigma, USA) at days 7, 14 and 21 with the medium exchange (30%) every 4 day according to the previously published method. To quantify glycosaminoglycan (GAG) deposition of the MSCs cultured in the HPAM3 for the predetermined days, dimethyl methyleneblue (DMMB, Sigma, USA) die was also usedin which a red color produced by DMMB binds to GAG and allows for the quantification of GAG [2].Firstly 10^6 MSCs were encapsulated into the 0.5 ml of the hydrogel with optimum concentration. Then cell/hydrogel constructs were put in the incubator for 7, 14, 21 days. 300 µl medium of each sample was taken off at each time interval and poured into a 2 ml vial; Then 1.2 ml (900 µl) acetone (Merck) was added to each vial and incubated at −20 °C for 24 hours. Subsequently, samples were centrifuged at 1800 rpm for 30 min at 4 °C, the supernatant was removed, and 1 ml papain digestion solution prepared by mixing 20 µg/ml papain. Then, 5 mM L-cysteine with PBS was added to the precipitated pellet, and the resulting samples were kept at 60 °C for 16 hours. Finally, the samples were boiled within 10–15 min and the GAG content was quantified using ELISA plate reader at 545 nm. The GAG content in the hydrogels was calculated by comparison the resulting fluorescence values with those of a standard curve obtained from known amounts of chondroitin sulfate (shark cartilage extract, Sigma, USA). As a positive control, chondrocytes were also encapsulated into the HPAM3 with the same condition.

III.2. Osteogenic evaluation. Alizarin Redstaining (ARS, Sigma–Aldrich) was performed to determine the presence of calcium deposition. The number of 5×10^2 cells in 500 µl culture medium were encapsulated (as a thin film) and cultured in a 6-well tissue culture polystyrene (TCPS) with the medium refreshing every three days. At 7, 14 and 21 days of culture, the cells were fixed in 4% paraformaldehyde and stained with alizarin red. The release of calcium was quantified as described previously by Gregory et al [3]**.** Briefly, HPAM6

hydrogels were removed from each well, and remaining cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and then incubated in ARS/PBS solution (2%, pH 4.2) for 45 min at room temperature. To remove any unbound ARS, cells rinsed five times with the NaCl solution (0.9%, Sigma, USA) prior to optical microscopy. Subsequently, 400 µl of 10 vol.% acetic acid solution was added to each well to dissolve the crystals. The absorbance reading was taken at 405 nm using an Eliza plate reader. The MSCs encapsulated in HPAM3, having no nHAp, also considered as negative control.

IV) Real Time-PCR analysis

Total RNA was extracted using RNeasy Plus mini kit (Qiagen) according to the manufacturer's instructions and quantified by a spectrophotometer instrument (Nanodrop). One strand DNA was generated using PrimeScript RT Reagent Kit. Real Time-PCR was performed using SYBR Premix Ex Taq II master mix (TaKaRa) with 7 different primers presented in Table S-IV.1 on One Step instrument (applied biosystem). Two various groups were applied for gene expression: 1) chondrogenic (Col I, Col II and Aggrecan), 2) osteogenic (Col I, Osteopontin and Runx2). For chondrogenic differentiation GAPDH was used as endogenous control, and for osteogenic differentiation all samples were normalized to Col I. Amplification cycles were applied to the mixtures at 95 ˚C for 15 sec, 95 ˚C for 5sec and 60 ˚C for 30 sec in each cycle. Fluorescence was collected in annealing-extension time in each cycle, and melting curve analysis carried out in three steps: 95 ˚C, 60 ˚C and stepwise heated to 95 ˚C.

Table S-IV.1 Primers used inchondrocyte and osteoblast gene expression

Primer	Sequence	Base Pair
GAPDH	Fw: CGTCTGCCCTATCAACTTTCG	102
	Rv: CGTTTCTCAGGCTCCCTCT	
Collagen type II	Fw: CAGGCAGAGGCAGGAAACTAAC	125
	Rv: CAGAGGTGTTTGACACGGAGTAG	
Aggrecan	Fw: ATGGCTTCCACCAGTGCG	127
	Rv: CGGATGCCGTAGGTTCTCA	
Collagen type I	Fw: GCGGTGGTTACGACTTTGGTT	140
	Rv: AGTGAGGAGGGTCTCAATCTG	
Osteopointin	Fw: GCAGAATCTCCTAACACCGCAG	132
	Rv: GGTCATCGTCCTCATCCTCATC	
Run x 2	Fw: GGAGTGGACGAGGCAAGAGT	169
	Rv: AGGCGGTCAGAGAACAAACTAGG	

V) Cell attachment

As the cell-matrix interaction happens during focal attachment and exerts an important effect on the cell differentiation [4-6], cell attachment to the hydrogels was examined. MSCs with a density of 1×10^5 cells per a 50 µl of culture medium were cultured on each hydrogel sample prepared on a cover glass ($1\times1cm^2$) and incubated at 37°C for 4 hours. Then, un-attached cells were washed four times with DMEM culture medium and counted using hemocytometer. Cover glass with no sample was also used as a negative control.Moreover, the morphology of MSCs encapsulated in the optimized hydrogels (HPAM3 and HPAM6) was studied using a scanning electron microscope (SEM) model HITACH S4160(Japan) at voltage of 15 KV. To prepare the samples for SEM analysis, after 3 days in vitro culturing,

the constructs were fixed with 4% formalin solution, dehydrated in serial alcohol solutions and sputter coated with gold.

Cell adhesion to the HPAM hydrogel may be attributed to the presence of negatively charged carboxylate groups on the HPAM backbone, which can contribute to the better protein absorption when it comes into contact with physiological fluids. Thevenot et al. and Keselowsky et al. reported that the presence of negative charges may promote adsorption of proteins which modulate cell adhesion and direct integrin binding [7, 8]. The results of the present study also indicated that cell adhesion was enhanced by the elevation of Cr (III) (Figure S-V.1). This may be attributed to the increase in gel stiffness by increasing the crosslink density. When the surface stiffness is increased, the extent of forces generated between cell and surrounding matrix would lead to the better cells spreading, proliferation as well as differentiation [9]. However, softer hydrogels with highly viscoelastic behavior could dissipate much of the received energy through molecular movements, so fewer signals would be transferred to the seeded cells. It was also demonstrated that the addition of nHAp as bioactive nano particles contributed to more cell attachment than the unfilled hydrogel(Figure S-V.1). This is probably due to the increase in the surface stiffness of the hydrogel. Furthermore, nanocrystalline hydroxyapatite in scaffolds could result in more biomimetic inspired constructs, which encourage greater cell adhesion [10].

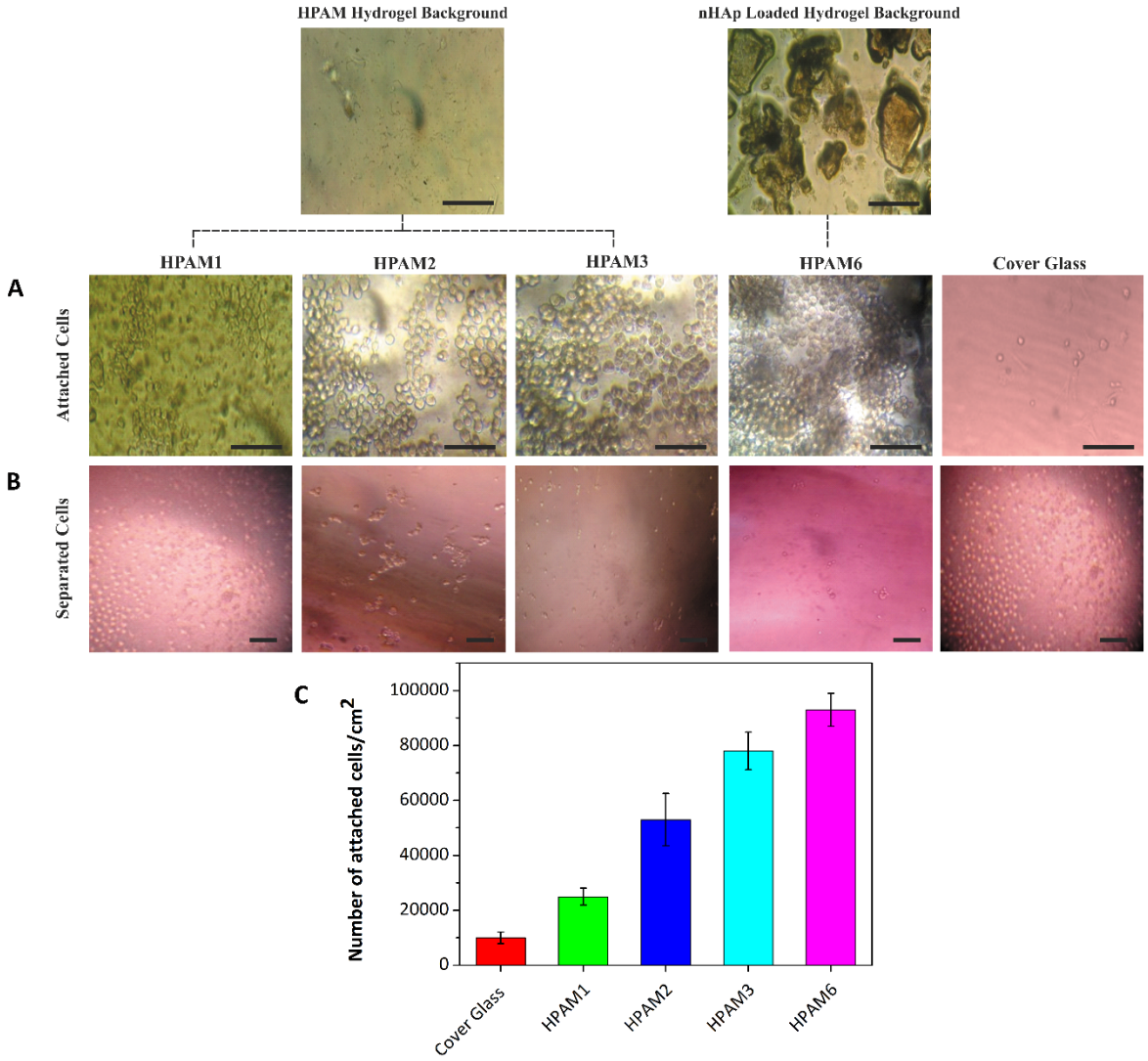

Fig. S-V.1 Determination of cell adhesion to HPAM hydrogels after 4 hrs. A) Representative microscopy images of cell attachment, B) Optical microscopy images of separated cells after washing the samples. C) Quantitative analysis of adherent cell number. Cell density: 1×10^5 cells/well (n=3) (Scale bar represent 100 µm).

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Tables and Figures' captions

Table. S-IV.1 Primers used in chondrocyte and osteoblast gene expression

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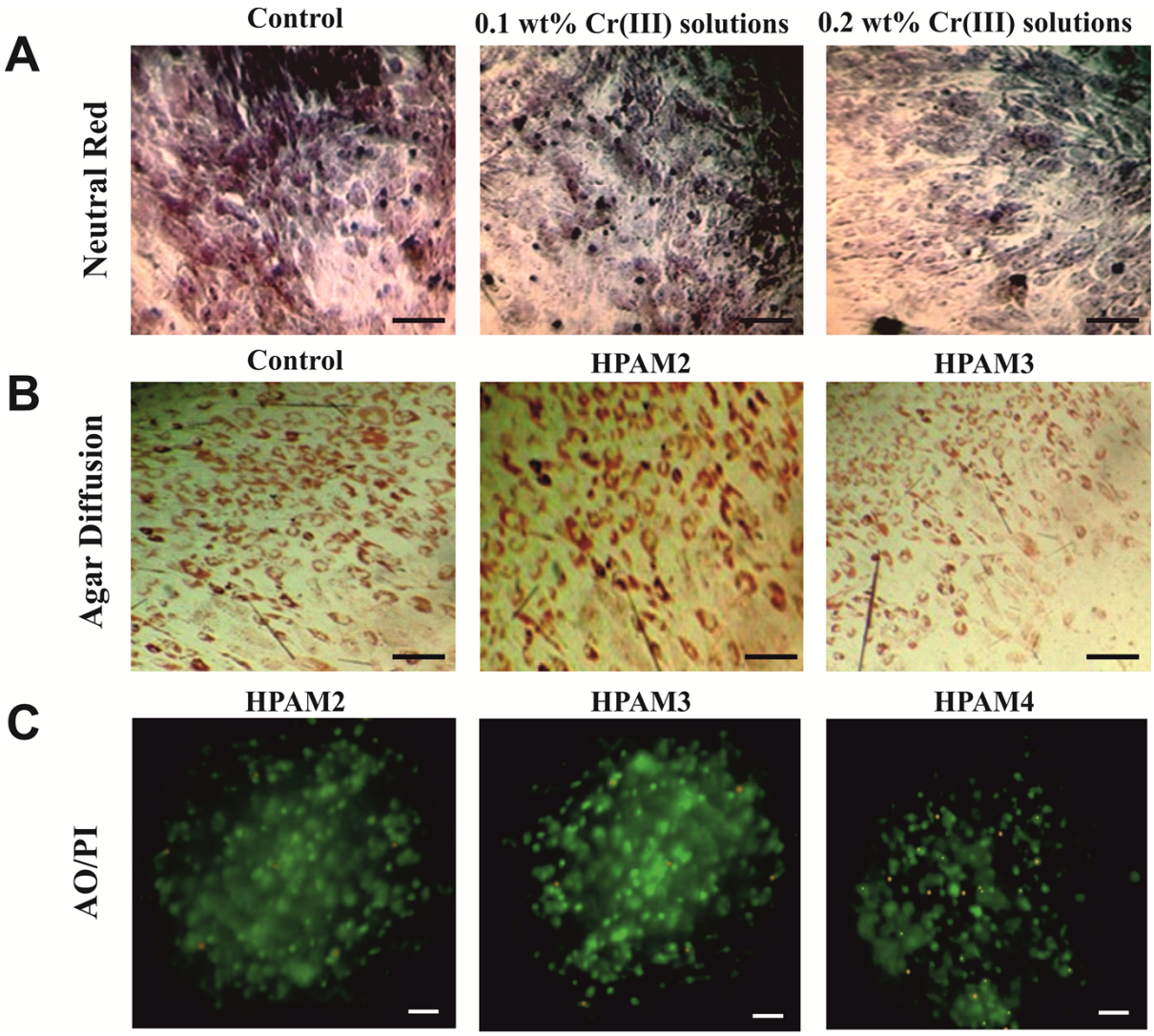

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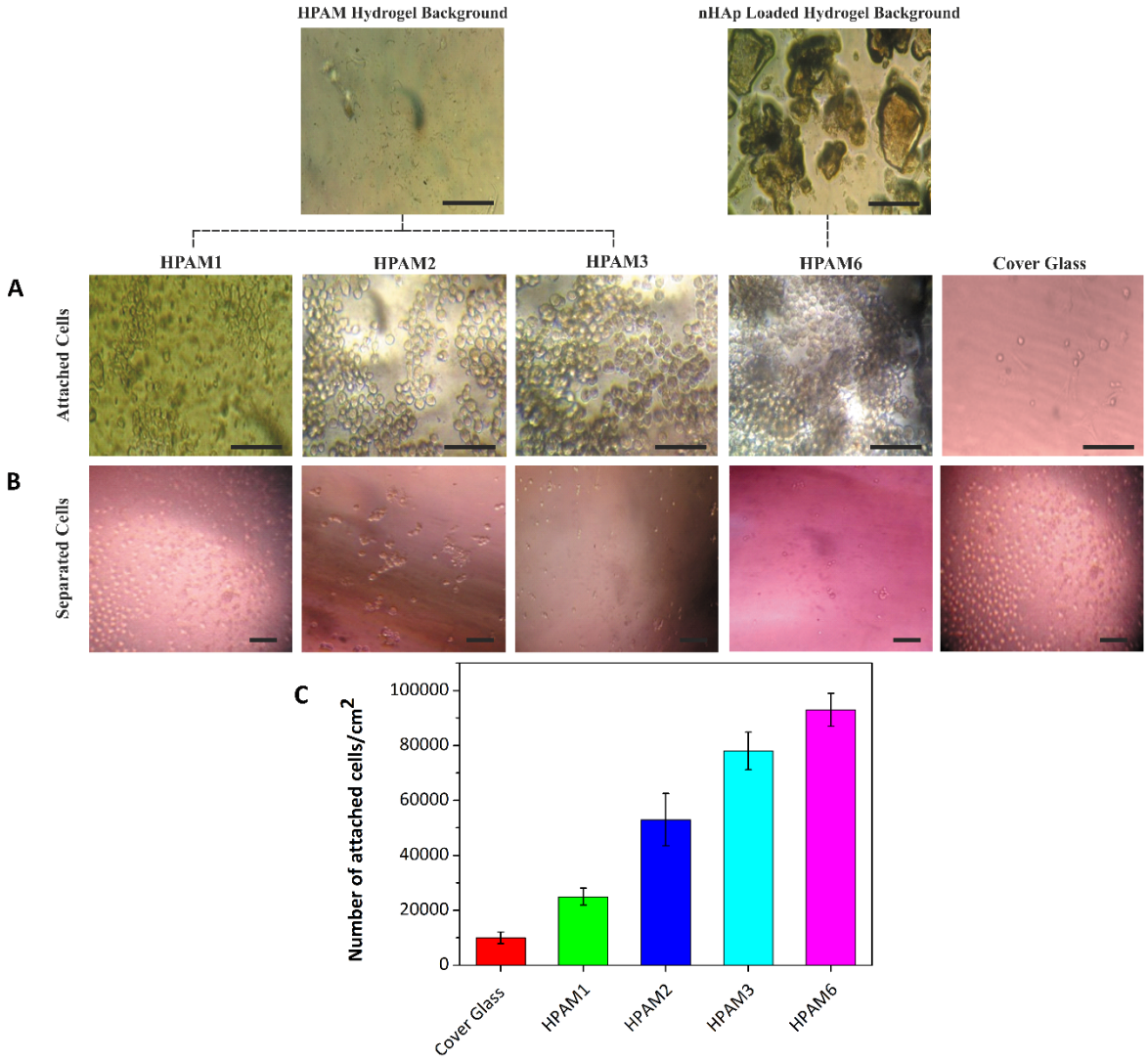

Fig. S-III.1 Determination of cell adhesion to HPAM hydrogels after 4 hrs. A) Representative microscopy images of cell attachment, B) Optical microscopy images of separated cells after washing the samples. C) Quantitative analysis of adherent cell number. Cell density: 1×10^5cells/well (n=3)(Scale bar represent 100 μm).