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Biosafety of a 3D-printed Intraocular Lens Made of a Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel *In Vitro* and *In Vivo*

Running title: Biosafety of Poly(Acrylamide-Co-Sodium Acrylate) as IOL material

Jiawen Li,^{1,2} Yijian Li,^{1,2} Xisu Hu,^{1,2} Yu Gong,^{1,2} Ben Bin Xu,³ Haiwei Xu,^{1,2} Zheng Qin Yin^{1,2}

¹Southwest Hospital/Southwest Eye Hospital, Third Military Medical University (Army Medical University), Chongqing, P.R. China

²Key Lab of Visual Damage and Regeneration & Restoration of Chongqing, Chongqing, P.R. China

³Smart Materials and Surfaces Lab, Faculty of Engineering and Environment, Northumbria University, Newcastle upon Tyne NE1 8ST, UK

Correspondence to: Haiwei Xu and Zheng Qin Yin. Southwest Hospital/Southwest Eye Hospital, Third Military Medical University (Army Medical University), Chongqing 400038, P.R. China.

E-mail addresses: xuhaiwei@tmmu.edu.cn; qinzyin@aliyun.com

Abstract

AIM: To assess the biosafety of a poly(acrylamide-co-sodium acrylate) hydrogel as a 3D-printed intraocular lens (IOL) material.

METHODS: The biosafety of the poly(acrylamide-co-sodium acrylate) hydrogel was first analyzed *in vitro* using human lens epithelial cells (LECs) and the ARPE19 cell line, and a CCK-8 assay was performed to investigate alterations in cell proliferation. A thin film of a poly(acrylamide-co-sodium acrylate) hydrogel and a conventional IOL were intraocularly implanted into the eyes of New Zealand white rabbits respectively, and a sham surgery served as control group. The anterior segment photographs, intraocular pressure (IOP), blood parameters and electroretinograms were recorded. Inflammatory cytokines in the aqueous humor, such as TNF α and IL-8, were examined by ELISA. Cell apoptosis of the retina was investigated by TUNEL assay, and macrophage activation was detected by immunostaining.

RESULTS: The poly(acrylamide-co-sodium acrylate) hydrogel did not slow cell proliferation when cocultured with human LECs or ARPE19 cells. The implantation of a thin film of a 3D-printed IOL composed of the poly(acrylamide-co-sodium acrylate) hydrogel did not affect the IOP, blood parameters, electroretinogram or optical structure in any of the three experimental groups (n=3 for each group). Both TNF α and IL-8 in the aqueous humor of the hydrogel group were transiently elevated 1 week post-operation and recovered to normal levels at 1 and 3 months post-operation. Iba1⁺ macrophages in the anterior chamber angle of the hydrogel group were increased markedly compared to those of the control group; however, there was no significant difference compared to those in the IOL group.

CONCLUSION: The poly(acrylamide-co-sodium acrylate) hydrogel is a safe material for 3D printing of personal IOLs that hold great potential for future clinical applications.

KEYWORDS: Cataract; Biosafety; Lens Epithelial Cells; ARPE19 Cells; Intraocular Lens; 3D Printing; Poly Hydrogel; Rabbit

INTRODUCTION

More than 95 million people worldwide have cataracts, which is the leading cause of blindness.¹ Phacoemulsification combined with intraocular lens (IOL) implantation is the most effective way to treat cataract so far.² However, the mass-produced model IOL based on a high-precision

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3 45 machine tool fails to meet the growing demands of personalized customization of the human lens,
4 46 and it is hard to obtain the best postoperative vision.³ Precision medicine is an inevitable trend in
5 47 the development of clinical medicine, personalized customization is an extremely important
6 48 component of precision medicine, and personalized IOL customization is an important means to
7 49 improve the outcome of cataract surgery.

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10 50 3D printing holds great potential in biomedical engineering research as well as in
11 51 ophthalmologic applications,^{4,6} which enables cost-effective products and instruments that aid in
12 52 therapeutic devices, such as IOLs, built specifically for individual cataract patients.⁷ Based on the
13 53 clinical data, it is possible for us to design a personalized IOL suitable for the size of the lens
14 54 capsule by digital optical modeling, simulating the quality of IOL imaging and correcting the
15 55 wave front aberration.⁸ Achievement of 3D printing of IOLs is a great challenge, and the
16 56 breakthrough bottleneck lies in the following points: 1) the fabrication of ultrahigh-precision
17 57 roughness of the IOL surface (micro/nano-precision);⁹ 2) the high transparency and UV-blocking
18 58 ability of materials; 3) the flexibility, water richness and micromechanical characteristics of the
19 59 organic materials; and 4) the biosafety of the material for 3D-printed IOLs.^{10,11}

20 60 Additive manufacturing and 3D printing create new approaches for the design and
21 61 manufacturing of implants, such as microstructured eye implants, including multifocal diffractive
22 62 aspheric IOL.¹² We planned to print the IOL mold with a new responsive
23 63 poly(acrylamide-co-sodium acrylate) hydrogel with 3D precise printing technology, which aims to
24 64 provide accurate and personalized IOL products for cataract patients. The gel is a fully diluted
25 65 crosslinking system that has no fluidity in the stable state. The main component of the gel is liquid
26 66 by weight. However, due to the 3D crosslinking network, the structural integrity of the hydrogel
27 67 network will not be dissolved due to the high hydrophilicity. Hydrogels are highly absorbent and
28 68 light permeable (containing more than 90% water). Due to its high water content, the hydrogel
29 69 also has a very similar flexibility to natural tissue, which makes it an ideal 3D printing material for
30 70 making personalized IOLs. However, as responsive smart materials, hydrogels can encapsulate
31 71 chemical compounds such as glucose, which are released by the stimulation of external factors,
32 72 such as changes in pH. Thus, whether a poly(acrylamide-co-sodium acrylate) hydrogel is toxic to
33 73 ocular tissue needs to be deliberately considered. Nevertheless, the issue of the biosafety of
34 74 poly(acrylamide-co-sodium acrylate) hydrogels is unclear so far.

35 75 Here, we utilized a poly(acrylamide-co-sodium acrylate) hydrogel to create a thin film in order to
36 76 investigate its biosafety after intraocular implantation. It was demonstrated *in vivo* that IOP, ERGs
37 77 and inflammatory factors in the aqueous humor exhibited no significant difference after
38 78 poly(acrylamide-co-sodium acrylate) hydrogel implantation. Moreover, similar results could be
39 79 verified by experiments *in vitro*. Taken together, the results indicate that the
40 80 poly(acrylamide-co-sodium acrylate) hydrogel does not harm the rabbit ocular tissue and causes
41 81 no inflammation *in vitro* or *in vivo*, supporting the idea that it is a safe material for on-demand
42 82 manufacturing of patient-personalized IOLs.

43 83 **MATERIALS AND METHODS**

44 84 **Laboratory Animal Welfare**

45 85 All experiments were performed on New Zealand white rabbits provided by the Experimental
46 86 Animal Centre of Southwest Hospital, Army Medical University. The New Zealand white rabbits
47 87 were all raised in the Animal Care Centre of the Army Medical University and maintained with
48 88 free access to water and food under a 12-h light/dark cycle. All animal experimental procedures

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3 89 were formally approved by the Institutional Review Board of the Army Medical University.
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5 90

6 91 **Preparation of the Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel Thin Film**

7 92 The hydrogel solution was prepared according to the following formula: 17.5%
8 93 N-isopropylacrylamide; 12.5% sodium acrylate; 1.5% N,N'-methylenebisacrylamide;
9 94 N,N,N',N'-tetramethylethylenediamine; 10% ammonium persulfate, H₂O. The solution was added
10 95 to one of the molds and immediately joined with another mold. Then, the two pieces of mold were
11 96 clamped together. After 30~60 minutes, after the gel was solidified, and the two molds were
12 97 removed. The formed hydrogel crystals were transferred to phosphate buffered saline (PBS) for
13 98 preservation.
14
15 99

100 **Intraocular Implantation of the Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel Thin Film** 101 **into New Zealand White Rabbit Eyes**

102 The New Zealand white rabbits were divided into three groups based on implants: the
103 poly(acrylamide-co-sodium acrylate) hydrogel thin film as the hydrogel group, the conventional
104 IOL as the IOL group, and the sham surgery as the control group (n=3 for each group). Animals
105 were anesthetized with 3% pentobarbital sodium 1 ml/kg through the ear-rim auricular vein; the
106 left eye was selected as the operative eye. Before the operation, tropicamide eye drops (Santen)
107 were used for mydriasis. The eyelid was opened with an eye speculum, and the conjunctival sac
108 was soaked with 5% povidone iodine for 90 seconds and then rinsed with saline. A 3.0 mm width
109 transparent corneal incision was made at the 12 o'clock position, a 1.0 mm width auxiliary corneal
110 incision was made at the 9 o'clock position, and sodium hyaluronate (SINGCLEAN) was injected
111 into the anterior chamber. For the control group, the incision was sutured directly with 10-0
112 polypropylene. For the hydrogel group and IOL group, we performed capsulorhexis, water
113 separation, and phacoemulsification of the lens nucleus (Laureate, Alcon, USA), cleared the lens
114 cortex with I/A, and injected the poly(acrylamide-co-sodium acrylate) hydrogel thin film (5 mm×5
115 mm) or IOL (860 UV, acrylic intraocular lenses as a positive control of biosafety). The 12 o'clock
116 incision was sutured with 10-0 nylon suture, balanced salt solution (BSS) was injected to form the
117 anterior chamber capacity, and tobramycin dexamethasone eye ointment (Alcon, USA) was
118 applied to the eyes after the operation. Intraocular pressures (IOP) was measured by a noncontact
119 tonometer at 1 day, 1 week, 1 month, and 3 months post-operation.
120

121 **Euthanasia and Histopathology**

122 Three months after implantation, the New Zealand white rabbits were euthanized with
123 pentobarbital sodium (400 mg/kg) administered intravenously. Afterwards, each eye containing
124 the implant was taken from each rabbit for histological examination. Specimens were fixed in 4%
125 paraformaldehyde for at least 24 hours and dehydrated in 30% sucrose for at least 24 hours,
126 followed by embedding with Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc., Torrance,
127 CA 90501, USA). All tissue pieces were cut into 2–4 μm thick tissue sections (Leica CM1900UV
128 cryostat). Then, all sections were routinely stained with hematoxylin-eosin (HE) and examined
129 under a light microscope (OLYMPUS DP71; Olympus Europa SE & Co. KG, Hamburg,
130 Germany) to assess fibroblast proliferation and anatomical abnormalities.
131

132 **Cell Culture**

1
2
3 133 Human lens epithelial cells (LECs) were expanded from lens explants, which were provided by
4 134 the Southwest Hospital Eye Bank. Briefly, the lens was washed in cold PBS containing 50 U mL⁻¹
5 135 penicillin and 100 µg mL⁻¹ streptomycin, cut into 5 mm×5 mm small pieces and seeded onto
6 136 human vitronectin (Gibco, Thermo Fisher, USA) precoated plates, and the explants were left for
7 137 attachment. The second day, LECs were cultured in conditioned medium consisting of
8 138 DMEM/F12 (HyClone), 10% FBS (Gibco), insulin-transferrin sodium selenite (1 µg mL⁻¹, Gibco),
9 139 penicillin (100 U mL⁻¹) and streptomycin (100 µg mL⁻¹, HyClone). As cells reached
10 140 subconfluence, they were gently passaged at 1:6 to expand. The ARPE19 cell line was cultured
11 141 according to the same procedures and conditions described above. The cells were co-cultured with
12 142 the poly(acrylamide-co-sodium acrylate) hydrogel thin film (5 mm×5 mm) or PBS as control.
13 143

14 144 **Immunostaining to Identify LECs**

15 145 Immunostaining was routinely performed as previously described.¹³ Sections were incubated
16 146 for 15 minutes with PBS, and sections were further incubated with PBS containing 0.1% (vol/vol)
17 147 Triton X-100 for 15 minutes and further blocked with 1% (wt/vol) bovine serum albumin (BSA)
18 148 in PBS at room temperature. The following primary antibodies were incubated at 4°C overnight:
19 149 Pax6 (1:500, Abcam), Sox2 (1:400, Abcam), αA-crystallin (1:200, Santa Cruz), α-SMA (1:400,
20 150 Abcam), and Iba1 (1:500, Wako).
21 151

22 152 **TUNEL Staining to Analyze the Apoptotic Cells and Immuno Cells in the Eyes Of Rabbits**

23 153 Using the TUNEL Assay Kit (Abcam) as previously described¹⁴, the secondary antibodies
24 154 included the goat anti-mouse Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 (Invitrogen).
25 155 DAPI (1:10, Beyotime) was incubated for 8-10 minutes at 20°C to counterstain nuclei, followed
26 156 by cover-slipping with antifade mounting medium (Beyotime).
27 157

28 158 **CCK-8 Assay to Analyze the Proliferation of LECs and APRE19 Cells**

29 159 The cytotoxicity of the poly(acrylamide-co-sodium acrylate) hydrogel was measured using the
30 160 Cell Counting Kit-8 (CCK-8) assay according to a previously published protocol.¹⁵ Absorbance at
31 161 wavelengths of 450 and 650 nm was collected using a microplate reader (Varioskan Flash,
32 162 Thermo Fisher, USA).
33 163

34 164 **ELISA Assay to Analyze Cytokines**

35 165 The aqueous humor was extracted with a 30G sterile needle syringe at the 9 o'clock position of
36 166 the limbus at 1 week, 1 month, and 3 months post-operation. ELISAs to detect IL-8 and TNF
37 167 (cloud-clone corp.) were performed as previously reported.¹⁶ The absorbance of each well was
38 168 read at 450 nm and 550 nm. The 550 nm values were subtracted from the 450 nm values to correct
39 169 for optical imperfections in the microplate.
40 170

41 171 **Electroretinogram Recording to Assess The Visual Function of Rabbits**

42 172 Scotopic flash electroretinogram recording was performed at 3 months post-operation as
43 173 described previously.¹⁷ Briefly, after 12-h dark adaptation, the rabbits were carefully anesthetized
44 174 with 3% pentobarbital sodium (1 ml/kg). The pupils were dilated with 1% tropicamide. The body
45 175 temperature was maintained at 37 °C with a heating pad to prevent hypothermia. Active gold
46 176 electrodes were placed onto cornea serving as the recording electrodes. The reference and ground
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177 electrodes were placed subcutaneously in the mid-frontal areas of the head and back, respectively.
 178 We performed light stimulation at densities of -2.5 , -0.5 , -0.02 , and $0.5 \log (\text{cd}^*\text{s}/\text{m}^2)$ for New
 179 Zealand white rabbits and $0.5 \log (\text{cd}^*\text{s}/\text{m}^2)$ for rabbits. The amplitudes and peak times of the
 180 a-waves and b-waves were recorded and processed through a RETI-Port device (Roland Consult).
 181 All experimental procedures were performed in a dark room under dim red safety light.

183 Microscopy, Image Acquisition, And Processing

184 All confocal images were collected by a Zeiss LSM880. Immunostaining data were all
 185 processed in ImageJ and Illustrator (Adobe).

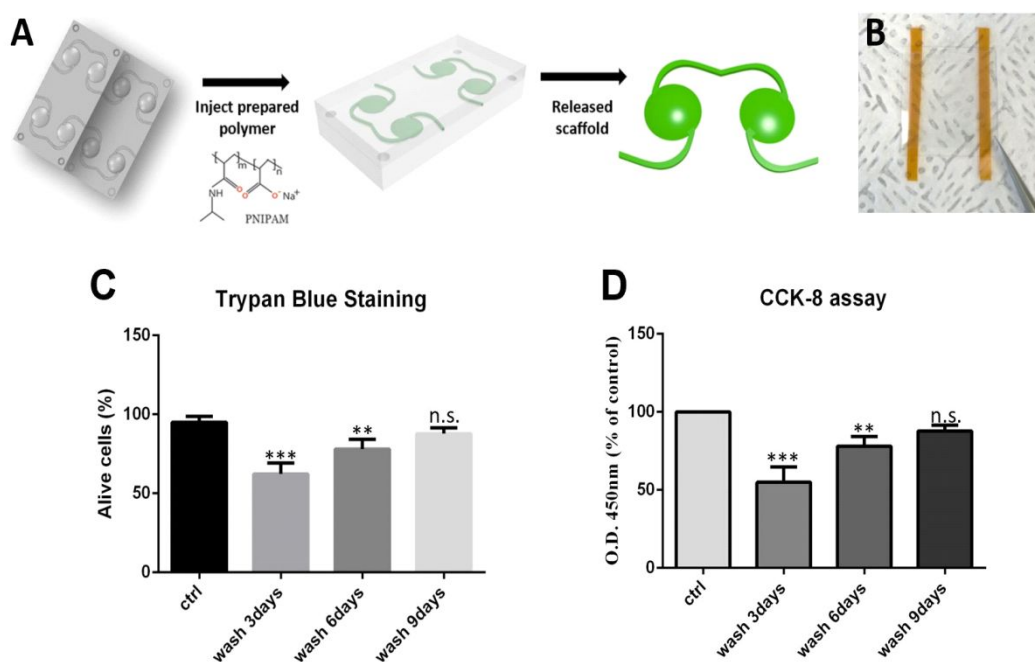
187 Statistical Analysis

188 Data are shown as the means \pm S.D. All statistical tests were performed by PRISM (GraphPad).
 189 Statistical significance was detected by Student's t test (parametric). A P -value <0.05 was
 190 considered statistically significant.

192 RESULTS

193 Synthesis of the Poly(acrylamide-co-sodium acrylate) Hydrogel

194 The new responsive poly(acrylamide-co-sodium acrylate) hydrogel was made from our
 195 modified formula as described in the materials and methods section. To produce patterned
 196 morphology and structure, a precise 3D-printed IOL mold was injected into the new responsive
 197 poly(acrylamide-co-sodium acrylate) hydrogel (Figure 1A). To better adapt for implantation into
 198 rabbit eyes, a thin film-like poly(acrylamide-co-sodium acrylate) hydrogel was made (Figure 1B).
 199 As a fresh hydrogel often remains unstable and releases small molecular chemical compounds
 200 such as glucose, to achieve a stable state of the poly(acrylamide-co-sodium acrylate) hydrogel, we
 201 pretreated the hydrogel with PBS and examined its optimal condition to reduce the influence on
 202 LECs. We found that after 9 days of pretreatment with PBS, the poly(acrylamide-co-sodium
 203 acrylate) hydrogel did not affect the survival or proliferation of LECs (Figure 1C, D).



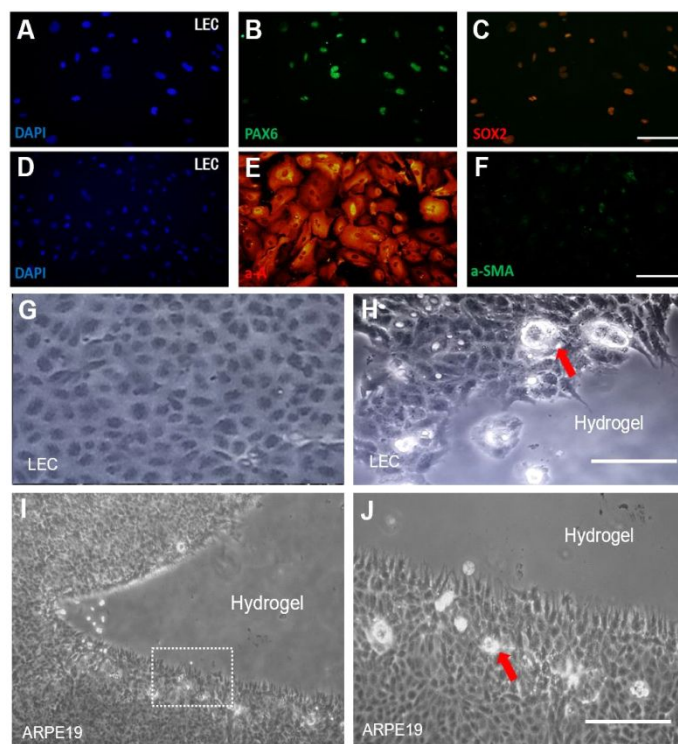
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206 **Figure 1 Synthesis of the poly(acrylamide-co-sodium acrylate) hydrogel thin film**

207 A: A precise 3D-printed IOL mold was injected into the new responsive poly(acrylamide-co-sodium acrylate)
 208 hydrogel to produce a patterned morphology and structure; B: Image of the poly(acrylamide-co-sodium acrylate)
 209 hydrogel thin film; C: Trypan blue staining of LECs co-cultured with the poly(acrylamide-co-sodium acrylate)
 210 hydrogel at 0, 3, 6, and 9 days of the PBS wash; D: CCK8 assay of LECs co-cultured with the
 211 poly(acrylamide-co-sodium acrylate) hydrogel at 0, 3, 6, and 9 days of the PBS wash; Data are shown as the
 212 mean \pm s.d. Independent sample t test. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.005$

214 **Effect of the Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel on LECs and ARPE19 Cells** 215 *in Vitro*

216 To investigate whether the poly(acrylamide-co-sodium acrylate) hydrogel disturbs cell viability,
 217 we cocultured the poly(acrylamide-co-sodium acrylate) hydrogel with LECs and ARPE19 cells.
 218 The cultured cells positively expressed LEC markers, such as PAX6 and SOX2, and synthesized
 219 the specific α -A crystallin and negatively expressed the fibroblast cell marker α -SMA, which
 220 identified the LECs (Figure 2A~F). When LECs were cocultured with the
 221 poly(acrylamide-co-sodium acrylate) hydrogel, cell apoptosis emerged only at the adjacent area
 222 around the hydrogel (Figure 2G, H). A similar result could be obtained when it cocultured with
 223 ARPE19 cells.
 224



225 **Figure 2 Coculture of poly(acrylamide-co-sodium acrylate) hydrogel thin films with LECs or ARPE19 cells**

226 A~F: Immunostaining of LECs showed that LECs were positively stained for the lens epithelial cell markers
 227 PAX6 (A, B), SOX2 (C), and α -A crystallin (D, E) and negatively stained for the fibroblast cell marker α -SMA
 228 (F); D~H: Bright field images of LECs co-cultured with (H) or without (G) the poly(acrylamide-co-sodium
 229 acrylate) hydrogel. Cells adjacent to the hydrogel were unable to attach to the matrix and ultimately underwent
 230 apoptosis (red arrow); I~J: Bright field images of ARPE19 cells co-cultured with the poly(acrylamide-co-sodium
 231 acrylate) hydrogel (I). The magnified image (J, white dashed area in I) shows that a minority of adjacent cells
 232

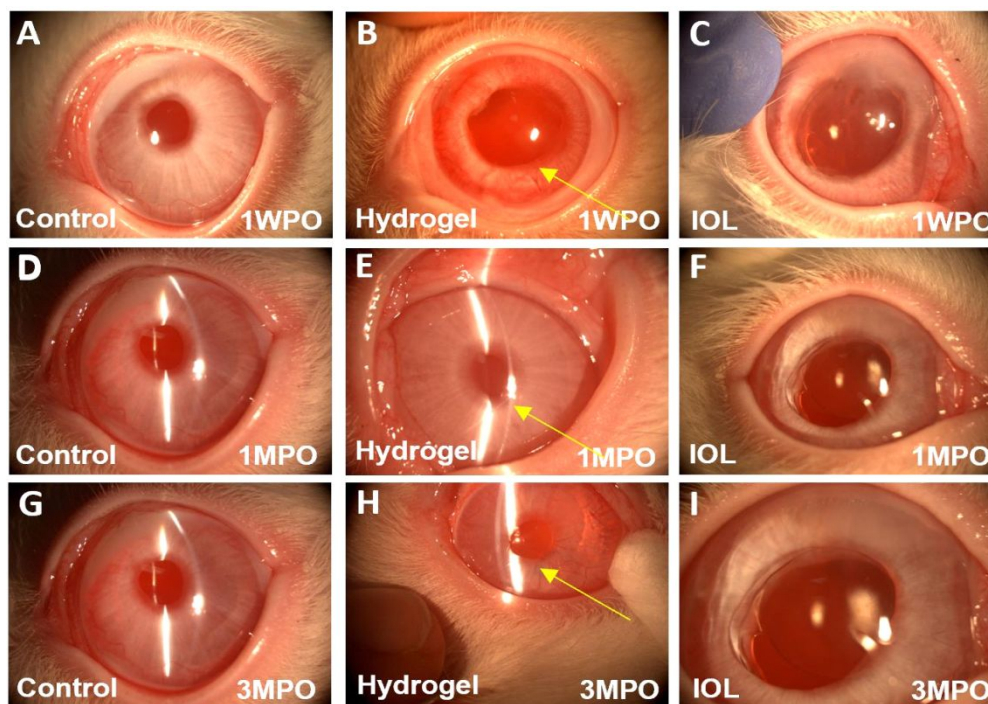
233 underwent cell apoptosis (red arrow), whereas the distal cells stayed in a normal proliferative state. Scale bar, 50
234 μm (A~F), 100 μm (G~J).

235

236 **Implantation of Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel into The Anterior** 237 **Chamber of New Zealand White Rabbits Does Not Induce Endophthalmitis**

238 To further evaluate the biosafety of poly(acrylamide-co-sodium acrylate) hydrogels, we
239 performed intraocular implantation in New Zealand white rabbits. Slit-lamp imaging at 1 week
240 post-operation indicated corrected location of the hydrogel at the anterior chamber (Figure 3A~C).
241 No significant corneal opacification, keratic precipitate, hypopyon, or synechia was observed at 1
242 month post-operation among all the groups (Figure 3D~F). Moreover, no anatomic abnormalities
243 were observed at 3 months post-operation among all the groups (Figure 3G~I). In addition, the
244 alteration of IOP after hydrogel implantation was not statistically significant (Figure 4A). To
245 further investigate the effect of poly(acrylamide-co-sodium acrylate) hydrogel implantation on the
246 intraocular inflammatory reaction, we performed ELISA to examine the key inflammatory factors
247 IL-8 and TNF- α . ELISA of the aqueous humor showed that IL-8 (Figure 4B) and TNA- α (Figure
248 4C) of both the hydrogel and IOL groups were elevated at the first week post-operation, but that of
249 the hydrogel group stayed consistent with that of the control group at 1 month and 3 months
250 post-operation. In sum, implantation of the poly(acrylamide-co-sodium acrylate) hydrogel did not
251 induce any endophthalmitis in the New Zealand white rabbit eyes.

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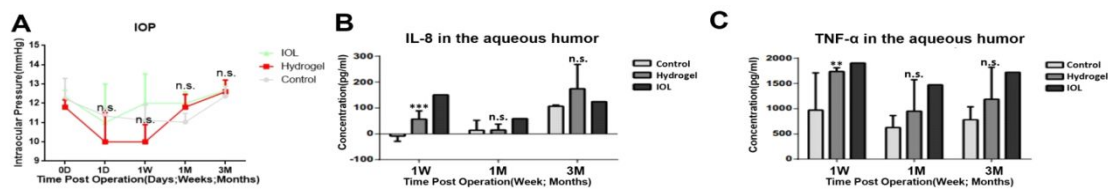
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254 **Figure 3 Implantation of poly(acrylamide-co-sodium acrylate) hydrogel thin film into the anterior chamber**
255 **of New Zealand white rabbits does not induce endophthalmitis**

256 A~C: Slit lamp images of the control group (A), hydrogel group (B) and IOL group (C) at 1 week post-operation
257 showed successful implantation and correct location of the implants; D~F: Slit lamp images of the control group
258 (D), hydrogel group (E) and IOL group (F) at 1 month post-operation showed no significant corneal opacification,
259 keratic precipitate or endophthalmitis; G~I: Slit lamp images of the control group (G), hydrogel group (H) and IOL

60

group (I) at 3 months post-operation showed no significant corneal opacification, keratic precipitate or endophthalmitis. Week post-operation (WPO); Month post-operation (MPO); Yellow arrow: Hydrogel.



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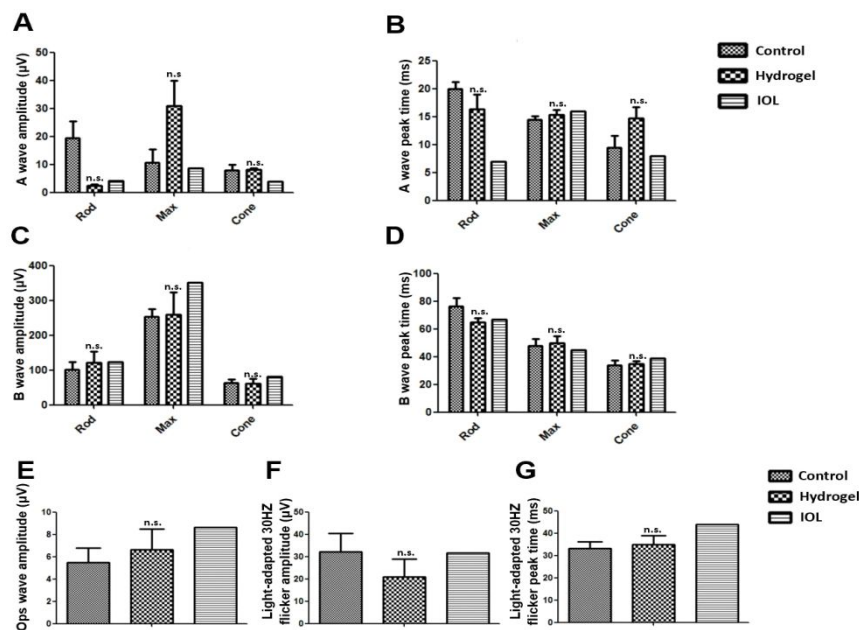
265 **Figure 4 Effects of poly(acrylamide-co-sodium acrylate) hydrogel implantation on the IOP and**
 266 **inflammatory factors in the aqueous humor**

267 A: The IOP recordings revealed showed no significant differences among the control, hydrogel and IOL groups;
 268 B~C: ELISA of inflammatory factors such as IL-8 (B) and TNF- α (C) in the aqueous humor of the control,
 269 hydrogel and IOL groups; Data are shown as the mean \pm s.d. Independent sample t test. * $p < 0.05$, ** $p < 0.01$; *** p
 270 < 0.005 ; n.s. no significance.

271

272 **Implantation of Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel Does Not Impair Visual** 273 **Function**

274 Visual function may be well reflected by visual electrophysiology examinations, such as
 275 electroretinography (ERG). To further investigate the effect of poly(acrylamide-co-sodium
 276 acrylate) hydrogel implantation on visual function, we conducted flash-ERG examinations of the
 277 control, hydrogel, and IOL groups. The amplitude of the a-wave of rod, cone and combined
 278 rod-cone (Max) ERG 3 months after hydrogel implantation was not significantly altered, nor was
 279 the peak time of the wave (Figure 5A, B). Similarly, both the amplitude and peak time of the
 280 b-wave of rod, cone and combined rod-cone (Max) ERG 3 months after hydrogel implantation
 281 were also not significantly altered (Figure 5C, D). These results indicated normal function of
 282 photoreceptor cells and bipolar cells in the hydrogel groups 3 months post-operation. Moreover,
 283 we analyzed the characteristics of the Ops wave and light-adapted 30 Hz flicker. Consistent with
 284 the results above, the implantation of hydrogel did not affect the amplitude of the Ops wave and
 285 light-adapted 30 Hz flicker, as well as its peak time (Figure 5E~G), indicating an intact and
 286 well-functioning inner retina and cone system.



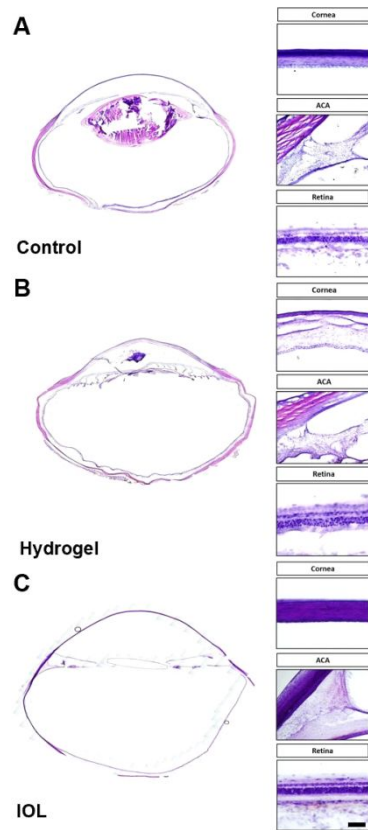
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288 **Figure 5 Flash-ERGs of New Zealand white rabbits at 3 months post-implantation**

289 A~B: Statistical analysis of the amplitudes (A) and peak time (B) of fERG a-waves at 0.5 log(cd*s/m²) in the three
 290 groups at 3 months post-operation; C~D: Statistical analysis of the amplitudes (C) and peak time (D) of fERG
 291 b-waves at 0.5 log(cd*s/m²) in the three groups at 3 months post-operation; E~G: Statistical analysis of the Ops
 292 wave amplitude (E) at 3.0 log(cd*s/m²) in the three groups at 3 months post-operation; F~G: Statistical analysis of
 293 the light-adapted 30 Hz flicker amplitude (F) and peak time (G) at 3.0 log(cd*s/m²) in the three groups at 3 months
 294 post-operation; Data are shown as the mean±s.d. Independent sample t test. *p < 0.05, **p < 0.01; ***p < 0.005;
 295 n.s. no significance.

296 **Implantation of Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel Does Not Damage Ocular** 297 **Histology**

298 Novel biomaterials are often toxic to the ocular tissue and cause severe abnormalities in
 299 histology. To evaluate the effect of poly(acrylamide-co-sodium acrylate) hydrogel implantation on
 300 the ocular histology, we first performed HE staining of New Zealand White Rabbit eyes after 3
 301 months of surgery. This result indicated that the hydrogel group maintained its normal histological
 302 characteristics, such as cell connections and layered lamination, as well as did the IOL group, all
 303 compared with the control group (Figure 6A, B, C). The TUNEL assay revealed that no cellular
 304 apoptosis emerged at the cornea, anterior chamber angle (ACA), or retina in any of the three
 305 groups, confirming the nontoxic character of the poly(acrylamide-co-sodium acrylate) hydrogel
 306 (Figure 7J~R). Moreover, IBA1⁺ macrophage cells did not accumulate in the cornea or retina
 307 except for the ACA in the hydrogel and IOL groups 3 months post-implantation (Figure 7A~I).
 308 The recruitment of macrophages at the ACA may represent the immune response caused by the
 309 hydrogel or IOL implants, which triggered the phagocytic activation of macrophages.



310

311 **Figure 6 The poly(acrylamide-co-sodium acrylate) hydrogel does not cause histological alteration 3 months**
 312 **post-implantation**

313 A: HE staining of New Zealand White Rabbit eyes showed the detailed histological identity of the cornea, anterior
 314 chamber angle, and retina in the control group; B: HE staining of New Zealand White Rabbit eyes showed the
 315 detailed histological identity of the cornea, anterior chamber angle, and retina in the hydrogel group. The cell
 316 connectivity and layered lamination were normal compared with those of the control group, even though the
 317 cornea was ruptured due to the limitations of the HE staining procedure; C: HE staining of New Zealand White
 318 Rabbit eyes showed the detailed histological identity of the cornea, anterior chamber angle, and retina in the IOL
 319 group. Scale bar, 100 μ m.

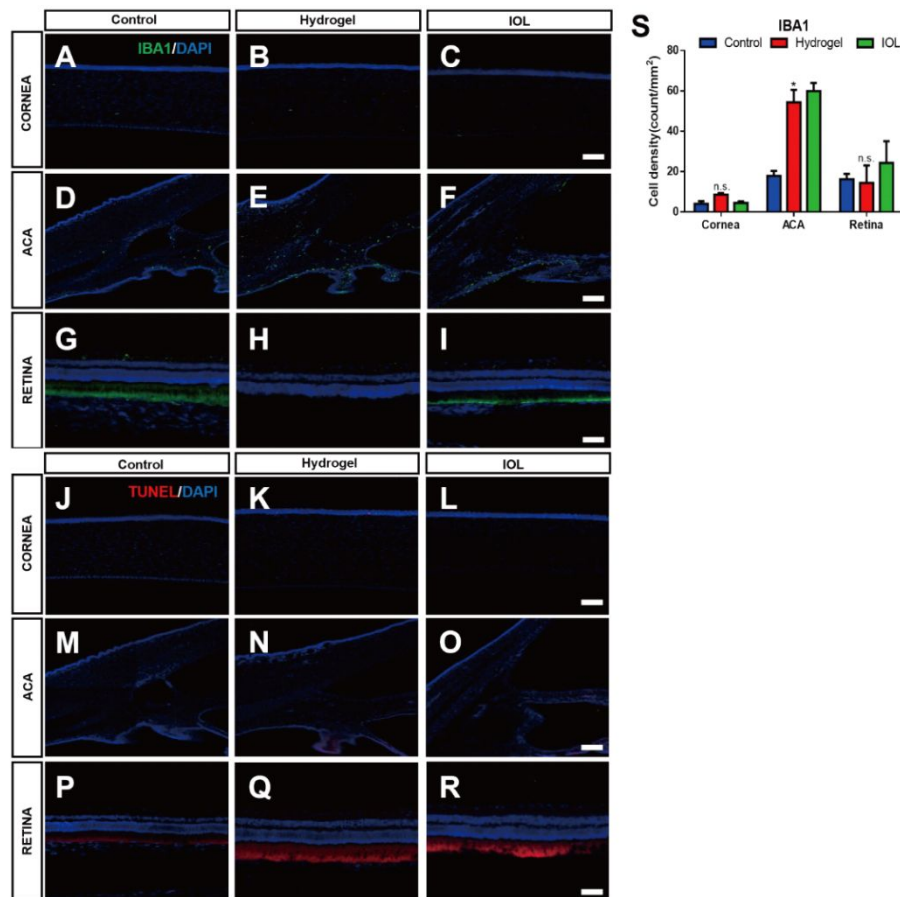


Figure 7 Macrophage activation and cell apoptosis of New Zealand white rabbit eyes 3 months after poly(acrylamide-co-sodium acrylate) hydrogel implantation

A~I: Immunostaining of Iba1 in New Zealand white rabbit eyes at 3 months post-implantation showed Iba1+ macrophage augmentation at the cornea (A~C), ACA (D~F) and retina (G~I) in the control group (A, D, G), the hydrogel group (G, E, H), and the IOL group (C,F,I); J~R: TUNEL assay of New Zealand White Rabbit eyes at 3 months post-implantation showed TUNEL+ apoptotic cell accumulation at the cornea (J~L), ACA (M~O) and retina (P~R) in the control group (J, M, P), the hydrogel group (K, N, Q), and the IOL group (L, O, R); S: Statistical analysis of Iba+ macrophages in the cornea, ANA, and retina in all three groups. Scale bar, 100 μ m. Data are shown as the mean \pm s.d. Independent sample t test. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.005$; n.s. no significance.

DISCUSSION

The eye exerts a unique ocular morphology, and all ocular organic architectures are susceptible to a number of diseases that may require treatment via different modalities, such as sustained drug delivery and artificial biotissue substitution.¹⁸ In the past century, hydrogels have been used in various applications as effective materials. The unique network structure of a hydrogel makes it highly hydrophilic and biocompatible, and it exhibits soft physical properties similarly to living tissue, which makes it an ideal and potential biomaterial for ophthalmic applications such as intraocular pumps, injections and implants, reducing comorbidities caused by glaucoma, cataracts, and diabetic retinopathies.¹⁹ 3D printing allows the creation of objects with complex three-dimensional geometries and photologies from a computer-aided design and printed biomaterials to tissue analog structures without any change in their mechanical or biological

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3 343 properties.^{20,21} However, given the nature of the polymers in hydrogel formulations and the
4 344 materials used in the preparation of ophthalmic gels, it is paramount that any new hydrogel
5 345 formulation intended for ocular application should be inevitably investigated for potential
6 346 toxicity/adverse effects on ocular tissues. In this report, we generated a
7 347 poly(acrylamide-co-sodium acrylate) hydrogel as a thin film and found that the newly formed
8 348 hydrogel needs 9 days of pretreatment with PBS to stabilize its structure, reflecting the
9 349 stimuli-controlled phase transition and small molecule release characteristics of
10 350 stimulus-responsive hydrogels²². Second, we performed coculture experiments *in vitro* and
11 351 implantation experiments *in vivo* to assess the effect of the hydrogel on ocular tissue. The
12 352 biosafety research parameters for structure, function, and inflammation, such as cell proliferation
13 353 assays, inflammation detection by slit lamp and aqueous humor tests, visual function assay by
14 354 electroretinogram, and anatomical abnormality detection by HE staining and immunostaining, all
15 355 indicated that the poly(acrylamide-co-sodium acrylate) hydrogel is qualified as a biosafe
16 356 clinical-grade biomaterial for 3D-printed IOLs by systematic investigation and verification both *in*
17 357 *vivo* and *in vitro*.

18 358 The polymers in preformed ophthalmic hydrogels could be semisynthetic or natural. Some of
19 359 them can transit from sol to gel conversely triggered by environmental stimuli such as
20 360 temperature, pH and ion concentration^{11,23}. As the poly(acrylamide-co-sodium acrylate) hydrogel
21 361 was primarily performed by 3D printing, it was harmful to cell proliferation when co-cultured
22 362 with LECs. (Figure 1C, D). However, pretreatment of the poly(acrylamide-co-sodium acrylate)
23 363 hydrogel with PBS for 9 days robustly reduced the toxic effect to LECs. This result indicated two
24 364 points of view: first, the primary poly(acrylamide-co-sodium acrylate) hydrogel could respond to
25 365 the cell culture environment conditions and release polymers that inhibit cellular proliferation at a
26 366 high concentration; second, adaptation to the extracellular environment is needed and should be
27 367 properly investigated and standardized for the safe clinical use of poly(acrylamide-co-sodium
28 368 acrylate) hydrogels.

29 369 When co-cultured with the poly(acrylamide-co-sodium acrylate) hydrogel, both LECs and
30 370 ARPE19 cells exhibit contact cell death, while cells located far away from the hydrogel grow
31 371 without any abnormalities. Cell migration and proliferation are highly dependent on the
32 372 extracellular matrix²⁴. To serve as an ideal environment and matrix, ophthalmic hydrogels must be
33 373 composed of gelatin²⁵, alginate²⁶, collagen²⁷, fibrin²⁸, and even molecularly modified biomaterials.
34 374 3D-printed IOLs have a high light transparency but may be populated by migratory and
35 375 proliferative cells^{29,30}. The contact cell death of the poly(acrylamide-co-sodium acrylate) hydrogel
36 376 prevented this possibility of this adverse effect of cell migration and proliferation, which makes it
37 377 an ideal transparent biomaterial with the least opacity after IOL implantation.

38 378 A previous study on the ocular tolerability of a new biomaterial formulation used the “Draize
39 379 rabbit eye irritation test,” which is the oldest and most classic test that has been employed to
40 380 evaluate potential ocular irritation. However, the Draize test is quite limited by its subjectivity,
41 381 poor reproducibility and the need for large numbers of live rabbits^{31,32}. In the current research, we
42 382 utilized ELISA to explore the inflammatory factor level of the aqueous humor, and we conducted
43 383 electroretinogram analyses to examine the visual function after hydrogel implantation. These
44 384 analyses well addressed the shortcomings of the Draize test and provided distinguished data to
45 385 gain insight into the effect on ocular inflammation and visual function.

46 386 Macrophages are a type of immune cell that engulfs and digests debris, foreign substances, and

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3 387 microbes³³. They are patrol guards in the microenvironment of all tissues, which are transformed
4 388 into active phagocytes by morphology and function and react quickly to various kinds of tissue
5 389 damage. There is conclusive evidence in animal models and in situ analysis of human tissues that
6 390 the macrophage response is a common feature of various retinal inflammatory diseases³⁴⁻³⁸. We
7 391 found accumulated Iba⁺ macrophages in the anterior chamber angle and iris of both hydrogel
8 392 groups 3 months post-operation. The morphology of Iba⁺ macrophages in the anterior chamber
9 393 angle and iris is amoeboid like, suggesting that the phagocytic amoeboid movement of emerging
10 394 macrophages is triggered by hydrogel implantation. Most importantly, we found similar results in
11 395 the IOL group, which served as a positive biosafe control group, and no corneal opacity, keratic
12 396 precipitate, hypopyon, or synechia were observed in the hydrogel or IOL group.

13 397 As the implanted IOL will be left in the eye for several years after surgery, there is growing
14 398 interest in the use of IOLs as drug reservoirs or as treatment methods for ophthalmic diseases.
15 399 Combining 3D printing technology and the biocompatible poly(acrylamide-co-sodium acrylate)
16 400 hydrogel as the printing material, as well as a potential material for stimuli-responsive hydrogel
17 401 fabrication, we hold the possibility of loading antibiotics, corticosteroids, and NSAIDs in the IOL,
18 402 which integrates cataract surgery and postoperative treatments as a one-step procedure³⁹.
19 403 Moreover, IOLs can also be incorporated with telescopic lenses, presbyopia-correcting lenses, or
20 404 accommodative polyfocal bioanalogical lenses^{40,41}. These advanced designs could become more
21 405 effective and personalized when combined with 3D printing and biocompatible hydrogels.
22 406 However, all these IOLs inevitably require rigid biosafety assessments and more clinical studies to
23 407 ascertain their safety and effectiveness.

24 408 In sum, the implantation of the poly(acrylamide-co-sodium acrylate) hydrogel did not disturb
25 409 the IOP, blood parameters, electroretinogram or optical structure, indicating that it will not cause
26 410 any ocular irritation. However, it is not clear whether this hydrogel lens maintains the nano
27 411 smooth surface, which can hopefully to correct visual errors, including the defocusing and
28 412 astigmatism caused by traditional IOLs. We will improve the manufacturing process in the future
29 413 and test the correction capacity of this new lens in terms of visual quality. Our research provides
30 414 insight into the biosafety of poly(acrylamide-co-sodium acrylate) hydrogels in ocular tissue and
31 415 demonstrates that poly(acrylamide-co-sodium acrylate) hydrogels are a safe material for 3D
32 416 printing of personal IOLs, which hold great potential for future clinical applications.

33 417

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37 421 acrylate) hydrogels.

38 422 **Author's Contributions:**

39 423 Jiawen Li: Conception and design, collection and assembly of data, data analysis and
40 424 interpretation, manuscript writing.

41 425 Yijian Li: Collection and assembly of data, data analysis and interpretation

42 426 Xisu Hu: Collection and assembly of data

43 427 Yu Gong: Collection and assembly of data, data analysis and interpretation

44 428 Ben Bin Xu: Design and collection mould of 3D IOL

45 429 Haiwei Xu: Critical reading and revision, administrative support, financial support.

46 430 Zheng Qin Yin: Financial support, final approval of the manuscript, critical reading and revision.

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7 435 **Conflicts of Interest:**

8 436 **Jiawen Li**, None; **Yijian Li**, None; **Xisu Hu**, None; **Yu Gong**, None; **Ben Bin Xu**, None; **Haiwei**
9 437 **Xu**, None; **Zheng Qin Yin**, None.

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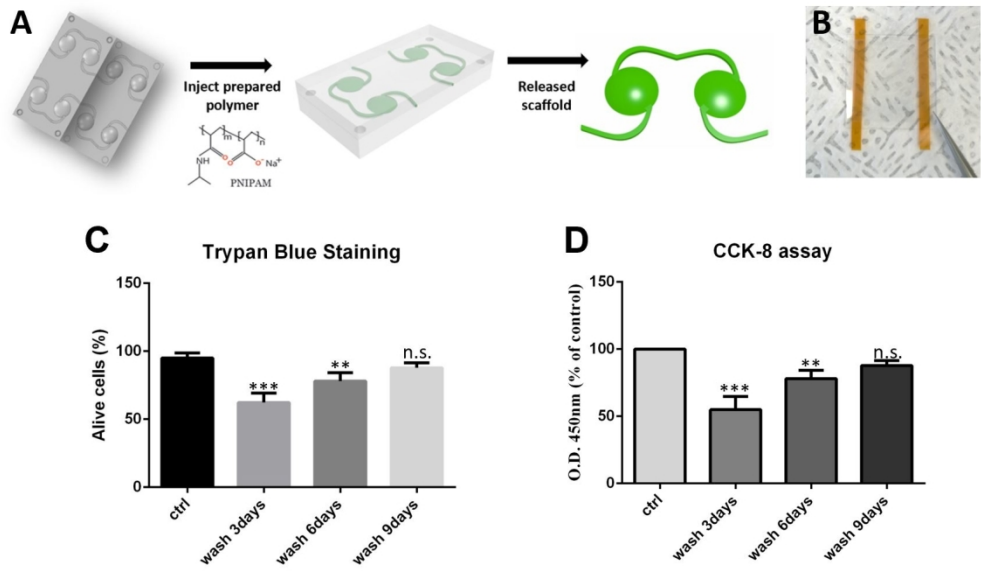


Figure1

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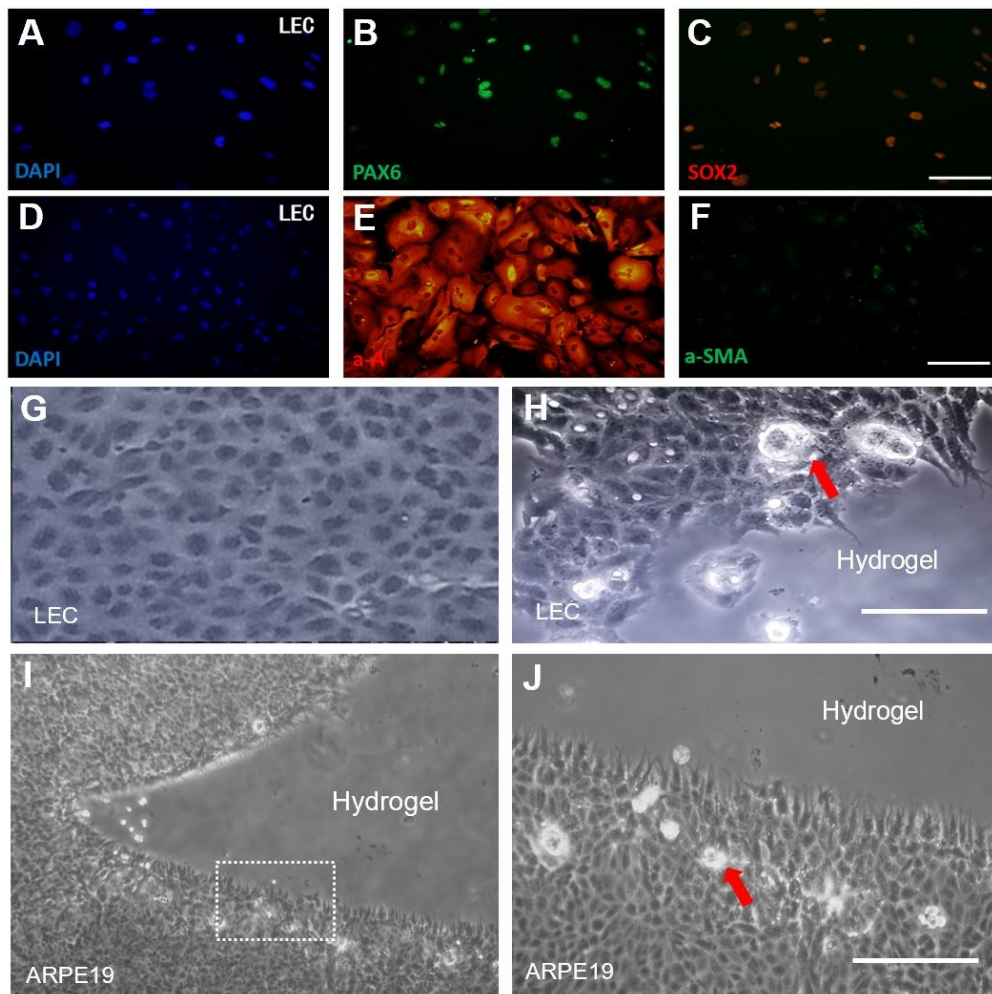


Figure2

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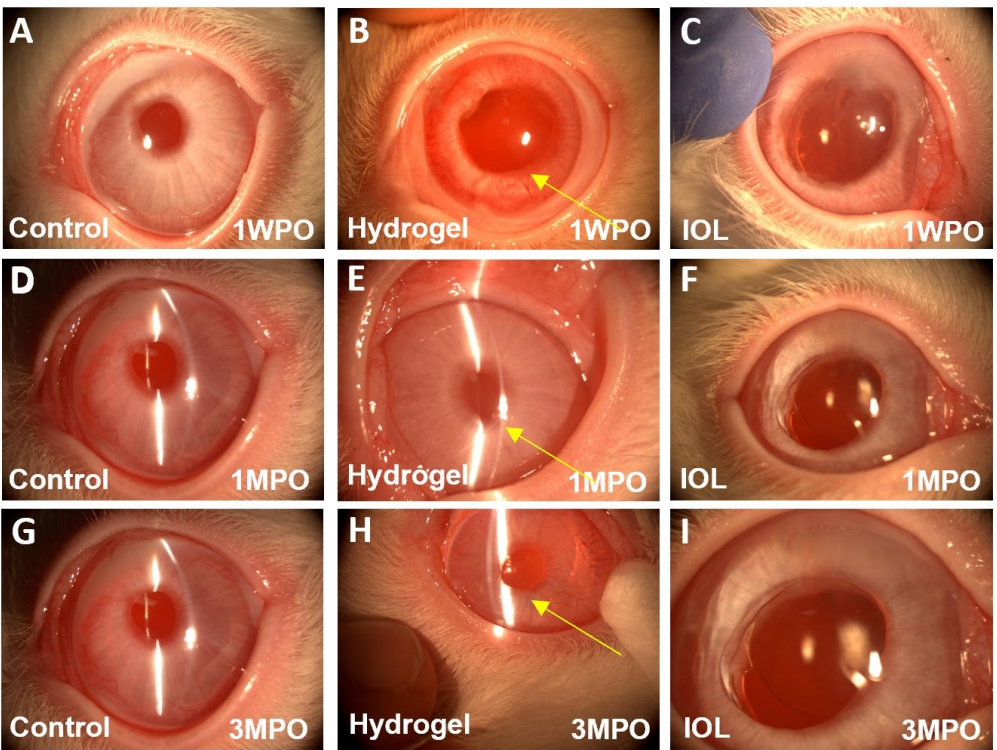


Figure3

155x117mm (216 x 216 DPI)

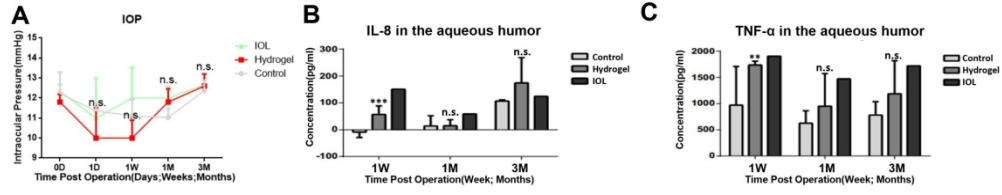


Figure 4

259x49mm (216 x 216 DPI)

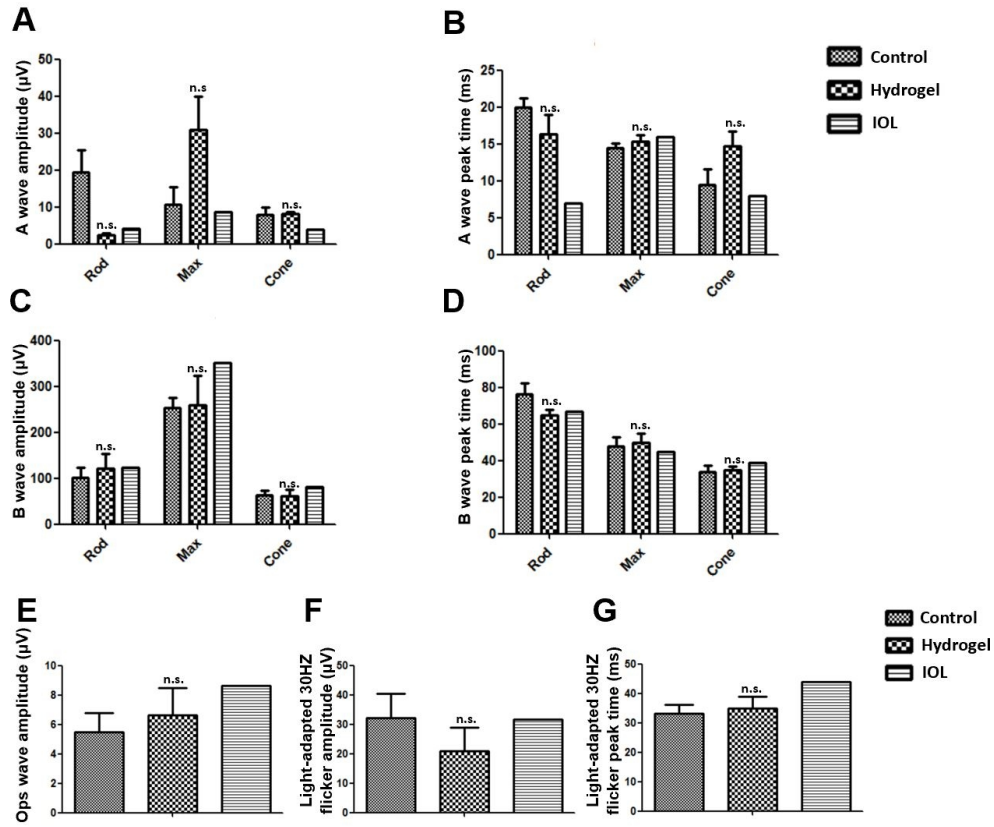


Figure5

150x125mm (216 x 216 DPI)

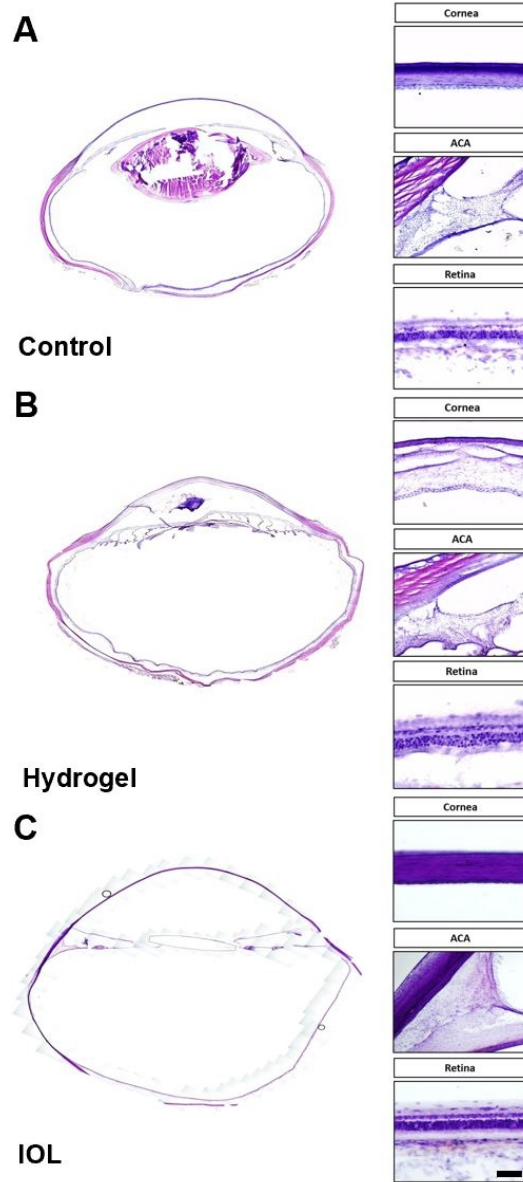


Figure6

59x133mm (216 x 216 DPI)

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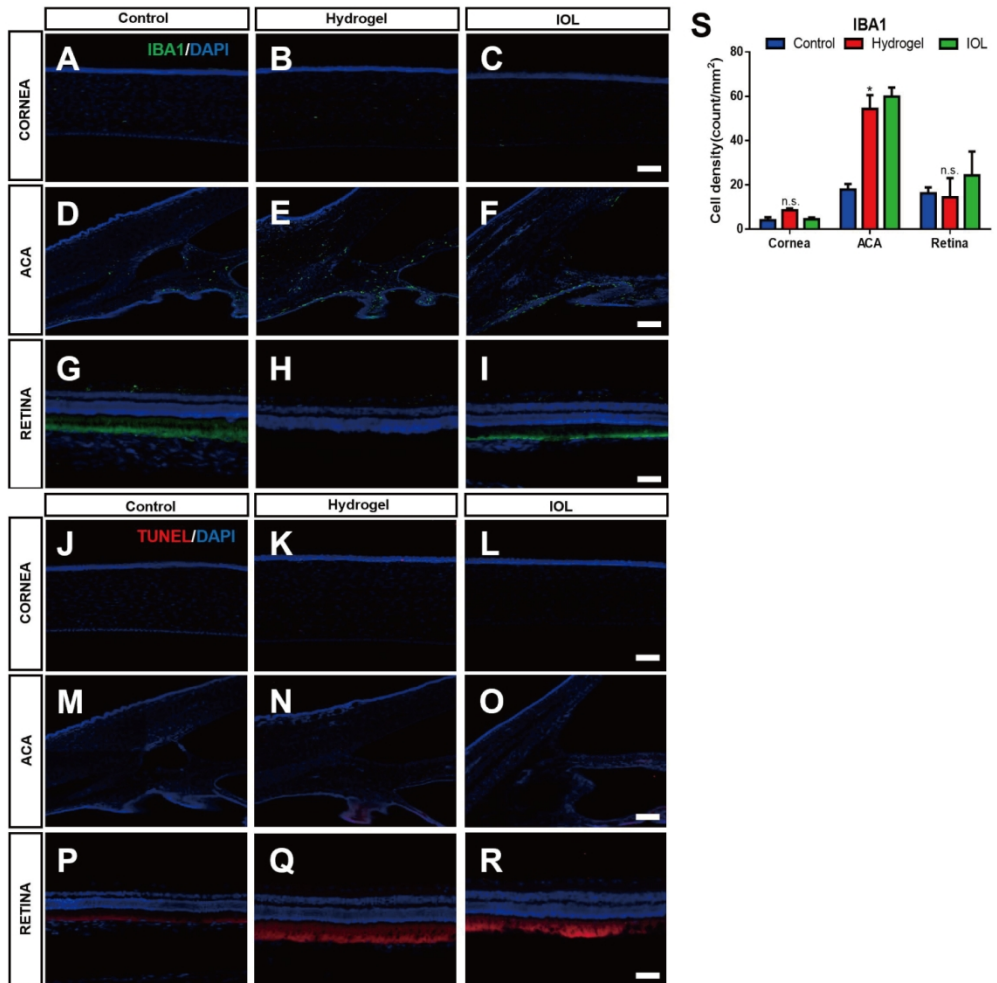


Figure7

Biosafety of a 3D-printed Intraocular Lens Made of a Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel *In Vitro* and *In Vivo*

Running title: Biosafety of Poly(Acrylamide-Co-Sodium Acrylate) as IOL material

Jiawen Li,^{1,2} Yijian Li,^{1,2} Xisu Hu,^{1,2} Yu Gong,^{1,2} Ben Bin Xu,³ Haiwei Xu,^{1,2} Zheng Qin Yin^{1,2}

¹Southwest Hospital/Southwest Eye Hospital, Third Military Medical University (Army Medical University), Chongqing, P.R. China

²Key Lab of Visual Damage and Regeneration & Restoration of Chongqing, Chongqing, P.R. China

³Smart Materials and Surfaces Lab, Faculty of Engineering and Environment, Northumbria University, Newcastle upon Tyne NE1 8ST, UK

Correspondence to: Haiwei Xu and Zheng Qin Yin. Southwest Hospital/Southwest Eye Hospital, Third Military Medical University (Army Medical University), Chongqing 400038, P.R. China.

E-mail addresses: xuhaiwei@tmmu.edu.cn; qinzyin@aliyun.com

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Author's Contributions:

Jiawen Li: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing. Yijian Li: Collection and assembly of data, data analysis and interpretation. Xisu Hu: Collection and assembly of data. Yu Gong: Collection and assembly of data, data analysis and interpretation. Ben Bin Xu: Design and collection mould of 3D IOL. Haiwei Xu: Critical reading and revision, administrative support, financial support. Zheng Qin Yin: Financial support, final approval of the manuscript, critical reading and revision.

Foundation:

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Conflicts of Interest:

Jiawen Li, None; **Yijian Li**, None; **Xisu Hu**, None; **Yu Gong**, None; **Ben Bin Xu**, None; **Haiwei Xu**, None; **Zheng Qin Yin**, None.

Abstract

AIM: To assess the biosafety of a poly(acrylamide-co-sodium acrylate) hydrogel as a 3D-printed intraocular lens (IOL) material.

METHODS: The biosafety of the poly(acrylamide-co-sodium acrylate) hydrogel was first analyzed *in vitro* using human lens epithelial cells (LECs) and the ARPE19 cell line, and a CCK-8 assay was performed to investigate alterations in cell proliferation. A thin film of a poly(acrylamide-co-sodium acrylate) hydrogel and a conventional IOL were intraocularly implanted into the eyes of New Zealand white rabbits respectively, and a sham surgery served as control group. The anterior segment photographs, intraocular pressure (IOP), blood parameters and electroretinograms were recorded. Inflammatory cytokines in the aqueous humor, such as TNF α and IL-8, were examined by ELISA. Cell apoptosis of the retina was investigated by TUNEL assay, and macrophage activation was detected by immunostaining.

RESULTS: The poly(acrylamide-co-sodium acrylate) hydrogel did not slow cell proliferation when cocultured with human LECs or ARPE19 cells. The implantation of a thin film of a 3D-printed IOL composed of the poly(acrylamide-co-sodium acrylate) hydrogel did not affect the IOP, blood parameters, electroretinogram or optical structure in any of the three experimental groups (n=3 for each group). Both TNF α and IL-8 in the aqueous humor of the hydrogel group were transiently elevated 1 week post-operation and recovered to normal levels at 1 and 3 months post-operation. Iba1⁺ macrophages in the anterior chamber angle of the hydrogel group were increased markedly compared to those of the control group; however, there was no significant difference compared to those in the IOL group.

CONCLUSION: The poly(acrylamide-co-sodium acrylate) hydrogel is a safe material for 3D printing of personal IOLs that hold great potential for future clinical applications.

KEYWORDS: Cataract; Biosafety; Lens Epithelial Cells; ARPE19 Cells; Intraocular Lens; 3D Printing; Poly Hydrogel; Rabbit

INTRODUCTION

More than 95 million people worldwide have cataracts, which is the leading cause of blindness.¹ Phacoemulsification combined with intraocular lens (IOL) implantation is the most effective way to treat cataract so far.² However, the mass-produced model IOL based on a high-precision machine tool fails to meet the growing demands of personalized customization of the human lens, and it is hard to obtain the best postoperative vision.³ Precision medicine is an inevitable trend in the development of clinical medicine, personalized customization is an extremely important component of precision medicine, and personalized IOL customization is an important means

1
2 to improve the outcome of cataract surgery.

3 3D printing holds great potential in biomedical engineering research as well as in ophthalmologic
4 applications,⁴⁻⁶ which enables cost-effective products and instruments that aid in therapeutic devices, such as
5 IOLs, built specifically for individual cataract patients.⁷ Based on the clinical data, it is possible for us to design a
6 personalized IOL suitable for the size of the lens capsule by digital optical modeling, simulating the quality of
7 IOL imaging and correcting the wave front aberration.⁸ Achievement of 3D printing of IOLs is a great challenge,
8 and the breakthrough bottleneck lies in the following points: 1) the fabrication of ultrahigh-precision roughness of
9 the IOL surface (micro/nano-precision);⁹ 2) the high transparency and UV-blocking ability of materials; 3) the
10 flexibility, water richness and micromechanical characteristics of the organic materials; and 4) the biosafety of the
11 material for 3D-printed IOLs.^{10,11}

12 Additive manufacturing and 3D printing create new approaches for the design and manufacturing of implants,
13 such as microstructured eye implants, including multifocal diffractive aspheric IOL.¹² We planned to print the IOL
14 mold with a new responsive poly(acrylamide-co-sodium acrylate) hydrogel with 3D precise printing technology,
15 which aims to provide accurate and personalized IOL products for cataract patients. The gel is a fully diluted
16 crosslinking system that has no fluidity in the stable state. The main component of the gel is liquid by weight.
17 However, due to the 3D crosslinking network, the structural integrity of the hydrogel network will not be
18 dissolved due to the high hydrophilicity. Hydrogels are highly absorbent and light permeable (containing more
19 than 90% water). Due to its high water content, the hydrogel also has a very similar flexibility to natural tissue,
20 which makes it an ideal 3D printing material for making personalized IOLs. However, as responsive smart
21 materials, hydrogels can encapsulate chemical compounds such as glucose, which are released by the stimulation
22 of external factors, such as changes in pH. Thus, whether a poly(acrylamide-co-sodium acrylate) hydrogel is toxic
23 to ocular tissue needs to be deliberately considered. Nevertheless, the issue of the biosafety of
24 poly(acrylamide-co-sodium acrylate) hydrogels is unclear so far.

25 Here, we utilized a poly(acrylamide-co-sodium acrylate) hydrogel to create a thin film in order to investigate its
26 biosafety after intraocular implantation. It was demonstrated *in vivo* that IOP, ERGs and inflammatory factors in
27 the aqueous humor exhibited no significant difference after poly(acrylamide-co-sodium acrylate) hydrogel
28 implantation. Moreover, similar results could be verified by experiments *in vitro*. Taken together, the results
29 indicate that the poly(acrylamide-co-sodium acrylate) hydrogel does not harm the rabbit ocular tissue and causes
30 no inflammation *in vitro* or *in vivo*, supporting the idea that it is a safe material for on-demand manufacturing of
31 patient-personalized IOLs.

32 MATERIALS AND METHODS

33 Laboratory Animal Welfare

34 All experiments were performed on New Zealand white rabbits provided by the Experimental Animal Centre of
35 Southwest Hospital, Army Medical University. The New Zealand white rabbits were all raised in the Animal Care
36 Centre of the Army Medical University and maintained with free access to water and food under a 12-h light/dark
37 cycle. All animal experimental procedures were formally approved by the Institutional Review Board of the Army
38 Medical University.

39 Preparation of the Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel Thin Film

40 The hydrogel solution was prepared according to the following formula: 17.5% N-isopropylacrylamide; 12.5%
41 sodium acrylate; 1.5% N,N'-methylenebisacrylamide; N,N,N',N'-tetramethylethylenediamine; 10% ammonium
42 persulfate, H₂O. The solution was added to one of the molds and immediately joined with another mold. Then, the
43 two pieces of mold were clamped together. After 30~60 minutes, after the gel was solidified, and the two molds
44 were removed. The formed hydrogel crystals were transferred to phosphate buffered saline (PBS) for preservation.

45 Intraocular Implantation of the Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel Thin Film into New 46 Zealand White Rabbit Eyes

47 The New Zealand white rabbits were divided into three groups based on implants: the
48 poly(acrylamide-co-sodium acrylate) hydrogel thin film as the hydrogel group, the conventional IOL as the IOL
49 group, and the sham surgery as the control group (n=3 for each group). Animals were anesthetized with 3%
50 pentobarbital sodium 1 ml/kg through the ear-rim auricular vein; the left eye was selected as the operative eye.
51 Before the operation, tropicamide eye drops (Santen) were used for mydriasis. The eyelid was opened with an eye
52 speculum, and the conjunctival sac was soaked with 5% povidone iodine for 90 seconds and then rinsed with
53 saline. A 3.0 mm width transparent corneal incision was made at the 12 o'clock position, a 1.0 mm width
54 auxiliary corneal incision was made at the 9 o'clock position, and sodium hyaluronate (SINGCLEAN) was
55 injected into the anterior chamber. For the control group, the incision was sutured directly with 10-0
56 polypropylene. For the hydrogel group and IOL group, we performed capsulorhexis, water separation, and
57 phacoemulsification of the lens nucleus (Laureate, Alcon, USA), cleared the lens cortex with I/A, and injected the
58 poly(acrylamide-co-sodium acrylate) hydrogel thin film (5 mm×5 mm) or IOL (860 UV, acrylic intraocular lenses
59 as a positive control of biosafety). The 12 o'clock incision was sutured with 10-0 nylon suture, balanced salt
60 solution (BSS) was injected to form the anterior chamber capacity, and tobramycin dexamethasone eye ointment
(Alcon, USA) was applied to the eyes after the operation. Intraocular pressures (IOP) was measured by a

1
2 noncontact tonometer at 1 day, 1 week, 1 month, and 3 months post-operation.
3

4 **Euthanasia and Histopathology**

5 Three months after implantation, the New Zealand white rabbits were euthanized with pentobarbital sodium
6 (400 mg/kg) administered intravenously. Afterwards, each eye containing the implant was taken from each rabbit
7 for histological examination. Specimens were fixed in 4% paraformaldehyde for at least 24 hours and dehydrated
8 in 30% sucrose for at least 24 hours, followed by embedding with Tissue-Tek O.C.T. Compound (Sakura Finetek
9 USA, Inc., Torrance, CA 90501, USA). All tissue pieces were cut into 2–4 μm thick tissue sections (Leica
10 CM1900UV cryostat). Then, all sections were routinely stained with hematoxylin-eosin (HE) and examined under
11 a light microscope (OLYMPUS DP71; Olympus Europa SE & Co. KG, Hamburg, Germany) to assess fibroblast
12 proliferation and anatomical abnormalities.
13

14 **Cell Culture**

15 Human lens epithelial cells (LECs) were expanded from lens explants, which were provided by the Southwest
16 Hospital Eye Bank. Briefly, the lens was washed in cold PBS containing 50 U mL⁻¹ penicillin and 100 μg mL⁻¹
17 streptomycin, cut into 5 mm \times 5 mm small pieces and seeded onto human vitronectin (Gibco, Thermo Fisher, USA)
18 precoated plates, and the explants were left for attachment. The second day, LECs were cultured in conditioned
19 medium consisting of DMEM/F12 (HyClone), 10% FBS (Gibco), insulin-transferrin sodium selenite (1 μg mL⁻¹,
20 Gibco), penicillin (100 U mL⁻¹) and streptomycin (100 μg mL⁻¹, HyClone). As cells reached subconfluence, they
21 were gently passaged at 1:6 to expand. The ARPE19 cell line was cultured according to the same procedures and
22 conditions described above. The cells were co-cultured with the poly(acrylamide-co-sodium acrylate) hydrogel
23 thin film (5 mm \times 5 mm) or PBS as control.
24

25 **Immunostaining to Identify LECs**

26 Immunostaining was routinely performed as previously described.¹³ Sections were incubated for 15 minutes
27 with PBS, and sections were further incubated with PBS containing 0.1% (vol/vol) Triton X-100 for 15 minutes
28 and further blocked with 1% (wt/vol) bovine serum albumin (BSA) in PBS at room temperature. The following
29 primary antibodies were incubated at 4°C overnight: Pax6 (1:500, Abcam), Sox2 (1:400, Abcam), α A-crystallin
30 (1:200, Santa Cruz), α -SMA (1:400, Abcam), and Iba1 (1:500, Wako).
31

32 **TUNEL Staining to Analyze the Apoptotic Cells and Immuno Cells in the Eyes Of Rabbits**

33 Using the TUNEL Assay Kit (Abcam) as previously described¹⁴, the secondary antibodies included the goat
34 anti-mouse Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 (Invitrogen). DAPI (1:10, Beyotime) was
35 incubated for 8-10 minutes at 20°C to counterstain nuclei, followed by cover-slipping with antifade mounting
36 medium (Beyotime).
37

38 **CCK-8 Assay to Analyze the Proliferation of LECs and APRE19 Cells**

39 The cytotoxicity of the poly(acrylamide-co-sodium acrylate) hydrogel was measured using the Cell Counting
40 Kit-8 (CCK-8) assay according to a previously published protocol.¹⁵ Absorbance at wavelengths of 450 and 650
41 nm was collected using a microplate reader (Varioskan Flash, Thermo Fisher, USA).
42

43 **ELISA Assay to Analyze Cytokines**

44 The aqueous humor was extracted with a 30G sterile needle syringe at the 9 o'clock position of the limbus at 1
45 week, 1 month, and 3 months post-operation. ELISAs to detect IL-8 and TNF (cloud-clone corp.) were performed
46 as previously reported.¹⁶ The absorbance of each well was read at 450 nm and 550 nm. The 550 nm values were
47 subtracted from the 450 nm values to correct for optical imperfections in the microplate.
48

49 **Electroretinogram Recording to Assess The Visual Function of Rabbits**

50 Scotopic flash electroretinogram recording was performed at 3 months post-operation as described previously.¹⁷
51 Briefly, after 12-h dark adaptation, the rabbits were carefully anesthetized with 3% pentobarbital sodium (1
52 ml/kg). The pupils were dilated with 1% tropicamide. The body temperature was maintained at 37 °C with a
53 heating pad to prevent hypothermia. Active gold electrodes were placed onto cornea serving as the recording
54 electrodes. The reference and ground electrodes were placed subcutaneously in the mid-frontal areas of the head
55 and back, respectively. We performed light stimulation at densities of -2.5, -0.5, -0.02, and 0.5 log (cd*s/m²) for
56 New Zealand white rabbits and 0.5 log (cd*s/m²) for rabbits. The amplitudes and peak times of the a-waves and
57 b-waves were recorded and processed through a RETI-Port device (Roland Consult). All experimental procedures
58 were performed in a dark room under dim red safety light.
59

60 **Microscopy, Image Acquisition, And Processing**

All confocal images were collected by a Zeiss LSM880. Immunostaining data were all processed in ImageJ and

Illustrator (Adobe).

Statistical Analysis

Data are shown as the means \pm S.D. All statistical tests were performed by PRISM (GraphPad). Statistical significance was detected by Student's t test (parametric). A P -value <0.05 was considered statistically significant.

RESULTS

Synthesis of the Poly(acrylamide-co-sodium acrylate) Hydrogel

The new responsive poly(acrylamide-co-sodium acrylate) hydrogel was made from our modified formula as described in the materials and methods section. To produce patterned morphology and structure, a precise 3D-printed IOL mold was injected into the new responsive poly(acrylamide-co-sodium acrylate) hydrogel (Figure 1A). To better adapt for implantation into rabbit eyes, a thin film-like poly(acrylamide-co-sodium acrylate) hydrogel was made (Figure 1B). As a fresh hydrogel often remains unstable and releases small molecular chemical compounds such as glucose, to achieve a stable state of the poly(acrylamide-co-sodium acrylate) hydrogel, we pretreated the hydrogel with PBS and examined its optimal condition to reduce the influence on LECs. We found that after 9 days of pretreatment with PBS, the poly(acrylamide-co-sodium acrylate) hydrogel did not affect the survival or proliferation of LECs (Figure 1C, D).

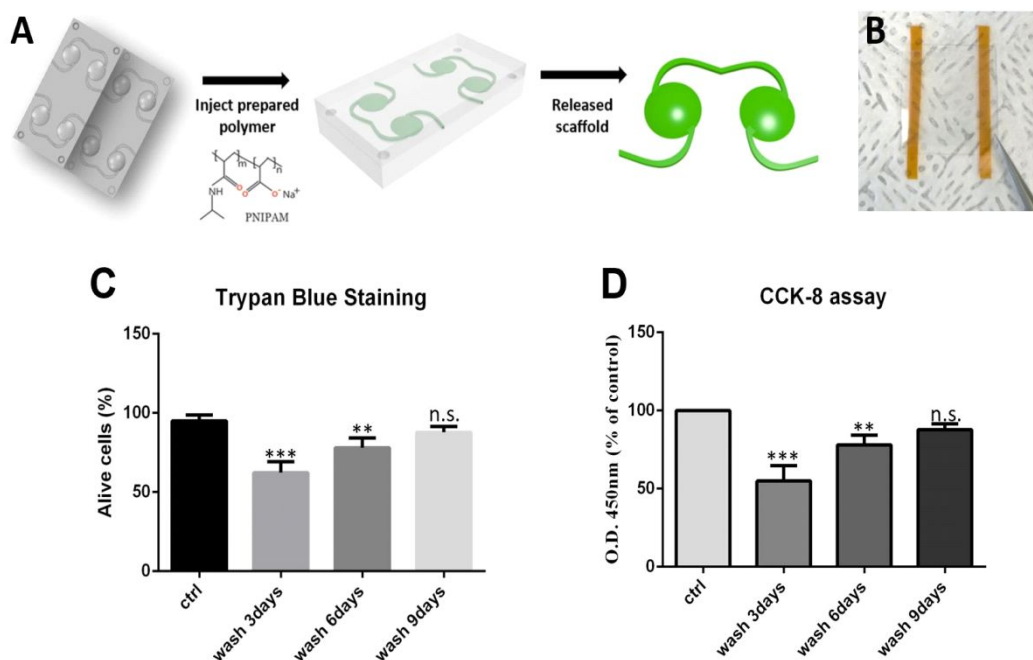


Figure 1 Synthesis of the poly(acrylamide-co-sodium acrylate) hydrogel thin film

A: A precise 3D-printed IOL mold was injected into the new responsive poly(acrylamide-co-sodium acrylate) hydrogel to produce a patterned morphology and structure; B: Image of the poly(acrylamide-co-sodium acrylate) hydrogel thin film; C: Trypan blue staining of LECs co-cultured with the poly(acrylamide-co-sodium acrylate) hydrogel at 0, 3, 6, and 9 days of the PBS wash; D: CCK8 assay of LECs co-cultured with the poly(acrylamide-co-sodium acrylate) hydrogel at 0, 3, 6, and 9 days of the PBS wash; Data are shown as the mean \pm s.d. Independent sample t test. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.005$

Effect of the Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel on LECs and ARPE19 Cells *in Vitro*

To investigate whether the poly(acrylamide-co-sodium acrylate) hydrogel disturbs cell viability, we cocultured the poly(acrylamide-co-sodium acrylate) hydrogel with LECs and ARPE19 cells. The cultured cells positively expressed LEC markers, such as PAX6 and SOX2, and synthesized the specific α -A crystallin and negatively expressed the fibroblast cell marker α -SMA, which identified the LECs (Figure 2A~F). When LECs were cocultured with the poly(acrylamide-co-sodium acrylate) hydrogel, cell apoptosis emerged only at the adjacent area around the hydrogel (Figure 2G, H). A similar result could be obtained when it cocultured with ARPE19 cells.

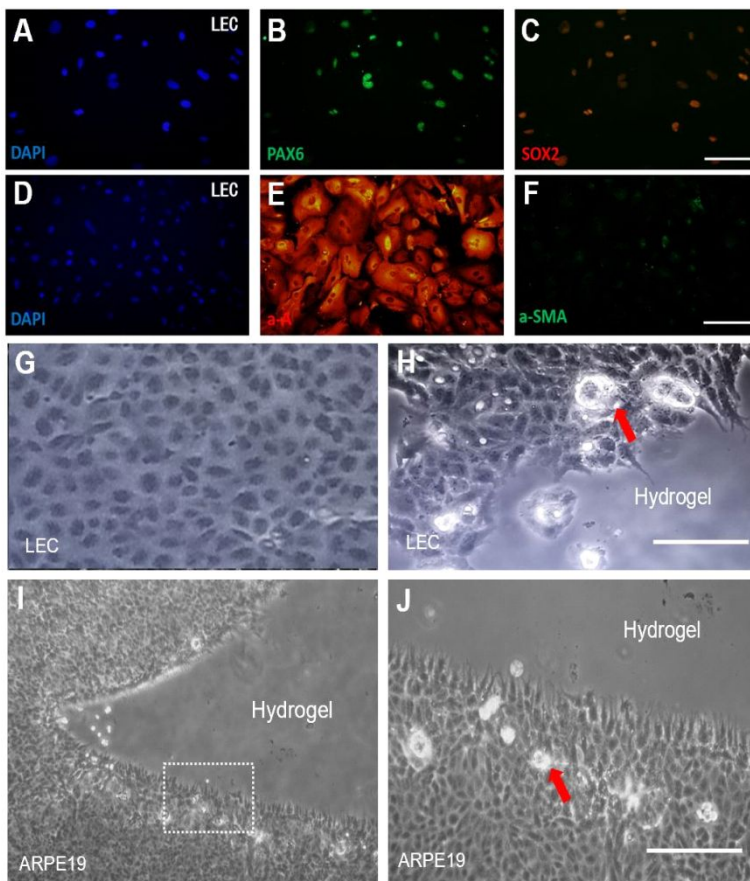


Figure 2 Coculture of poly(acrylamide-co-sodium acrylate) hydrogel thin films with LECs or ARPE19 cells
 A~F: Immunostaining of LECs showed that LECs were positively stained for the lens epithelial cell markers PAX6 (A, B), SOX2 (C), and α -A crystallin (D, E) and negatively stained for the fibroblast cell marker α -SMA (F); D~H: Bright field images of LECs co-cultured with (H) or without (G) the poly(acrylamide-co-sodium acrylate) hydrogel. Cells adjacent to the hydrogel were unable to attach to the matrix and ultimately underwent apoptosis (red arrow); I~J: Bright field images of ARPE19 cells co-cultured with the poly(acrylamide-co-sodium acrylate) hydrogel (I). The magnified image (J, white dashed area in I) shows that a minority of adjacent cells underwent cell apoptosis (red arrow), whereas the distal cells stayed in a normal proliferative state. Scale bar, 50 μ m (A~F), 100 μ m (G~J).

Implantation of Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel into The Anterior Chamber of New Zealand White Rabbits Does Not Induce Endophthalmitis

To further evaluate the biosafety of poly(acrylamide-co-sodium acrylate) hydrogels, we performed intraocular implantation in New Zealand white rabbits. Slit-lamp imaging at 1 week post-operation indicated corrected location of the hydrogel at the anterior chamber (Figure 3A~C). No significant corneal opacification, keratic precipitate, hypopyon, or synechia was observed at 1 month post-operation among all the groups (Figure 3D~F). Moreover, no anatomic abnormalities were observed at 3 months post-operation among all the groups (Figure 3G~I). In addition, the alteration of IOP after hydrogel implantation was not statistically significant (Figure 4A). To further investigate the effect of poly(acrylamide-co-sodium acrylate) hydrogel implantation on the intraocular inflammatory reaction, we performed ELISA to examine the key inflammatory factors IL-8 and TNF- α . ELISA of the aqueous humor showed that IL-8 (Figure 4B) and TNF- α (Figure 4C) of both the hydrogel and IOL groups were elevated at the first week post-operation, but that of the hydrogel group stayed consistent with that of the control group at 1 month and 3 months post-operation. In sum, implantation of the poly(acrylamide-co-sodium acrylate) hydrogel did not induce any endophthalmitis in the New Zealand white rabbit eyes.

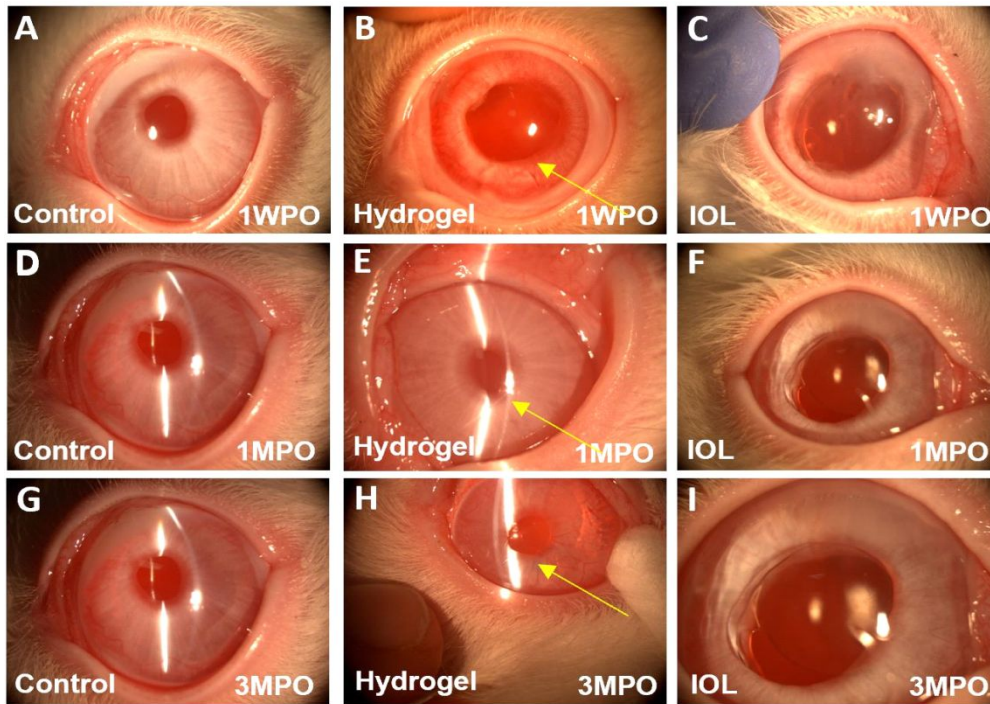


Figure 3 Implantation of poly(acrylamide-co-sodium acrylate) hydrogel thin film into the anterior chamber of New Zealand white rabbits does not induce endophthalmitis

A~C: Slit lamp images of the control group (A), hydrogel group (B) and IOL group (C) at 1 week post-operation showed successful implantation and correct location of the implants; D~F: Slit lamp images of the control group (D), hydrogel group (E) and IOL group (F) at 1 month post-operation showed no significant corneal opacification, keratic precipitate or endophthalmitis; G~I: Slit lamp images of the control group (G), hydrogel group (H) and IOL group (I) at 3 months post-operation showed no significant corneal opacification, keratic precipitate or endophthalmitis. Week post-operation (WPO); Month post-operation (MPO); Yellow arrow: Hydrogel.

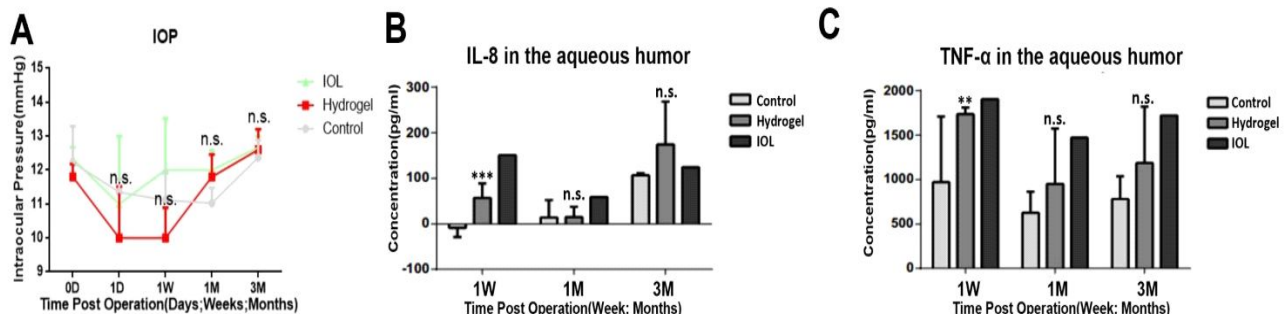


Figure 4 Effects of poly(acrylamide-co-sodium acrylate) hydrogel implantation on the IOP and inflammatory factors in the aqueous humor

A: The IOP recordings revealed showed no significant differences among the control, hydrogel and IOL groups; B~C: ELISA of inflammatory factors such as IL-8 (B) and TNF- α (C) in the aqueous humor of the control, hydrogel and IOL groups; Data are shown as the mean \pm s.d. Independent sample t test. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.005$; n.s. no significance.

Implantation of Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel Does Not Impair Visual Function

Visual function may be well reflected by visual electrophysiology examinations, such as electroretinography (ERG). To further investigate the effect of poly(acrylamide-co-sodium acrylate) hydrogel implantation on visual function, we conducted flash-ERG examinations of the control, hydrogel, and IOL groups. The amplitude of the a-wave of rod, cone and combined rod-cone (Max) ERG 3 months after hydrogel implantation was not significantly altered, nor was the peak time of the wave (Figure 5A, B). Similarly, both the amplitude and peak time of the b-wave of rod, cone and combined rod-cone (Max) ERG 3 months after hydrogel implantation were also not significantly altered (Figure 5C, D). These results indicated normal function of photoreceptor cells and bipolar cells in the hydrogel groups 3 months post-operation. Moreover, we analyzed the characteristics of the Ops wave and light-adapted 30 Hz flicker. Consistent with the results above, the implantation of hydrogel did not affect the amplitude of the Ops wave and light-adapted 30 Hz flicker, as well as its peak time (Figure 5E~G), indicating an intact and well-functioning inner retina and cone system.

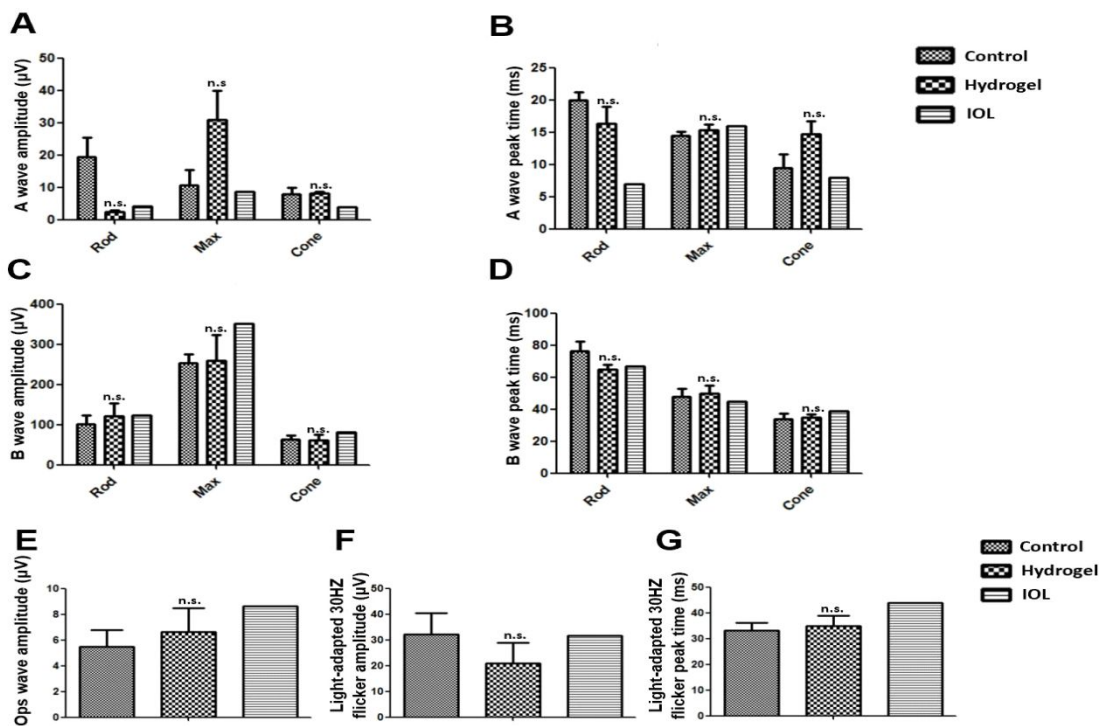


Figure 5 Flash-ERGs of New Zealand white rabbits at 3 months post-implantation

A~B: Statistical analysis of the amplitudes (A) and peak time (B) of fERG a-waves at $0.5 \log(\text{cd}^*\text{s}/\text{m}^2)$ in the three groups at 3 months post-operation; C~D: Statistical analysis of the amplitudes (C) and peak time (D) of fERG b-waves at $0.5 \log(\text{cd}^*\text{s}/\text{m}^2)$ in the three groups at 3 months post-operation; E~G: Statistical analysis of the Ops wave amplitude (E) at $3.0 \log(\text{cd}^*\text{s}/\text{m}^2)$ in the three groups at 3 months post-operation; F~G: Statistical analysis of the light-adapted 30 Hz flicker amplitude (F) and peak time (G) at $3.0 \log(\text{cd}^*\text{s}/\text{m}^2)$ in the three groups at 3 months post-operation; Data are shown as the mean \pm s.d. Independent sample t test. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.005$; n.s. no significance.

Implantation of Poly(Acrylamide-Co-Sodium Acrylate) Hydrogel Does Not Damage Ocular Histology

Novel biomaterials are often toxic to the ocular tissue and cause severe abnormalities in histology. To evaluate the effect of poly(acrylamide-co-sodium acrylate) hydrogel implantation on the ocular histology, we first performed HE staining of New Zealand White Rabbit eyes after 3 months of surgery. This result indicated that the hydrogel group maintained its normal histological characteristics, such as cell connections and layered lamination, as well as did the IOL group, all compared with the control group (Figure 6A, B, C). The TUNEL assay revealed that no cellular apoptosis emerged at the cornea, anterior chamber angle (ACA), or retina in any of the three groups, confirming the nontoxic character of the poly(acrylamide-co-sodium acrylate) hydrogel (Figure 7J~R). Moreover, IBA1⁺ macrophage cells did not accumulate in the cornea or retina except for the ACA in the hydrogel and IOL groups 3 months post-implantation (Figure 7A~I). The recruitment of macrophages at the ACA may represent the immune response caused by the hydrogel or IOL implants, which triggered the phagocytic activation of macrophages.

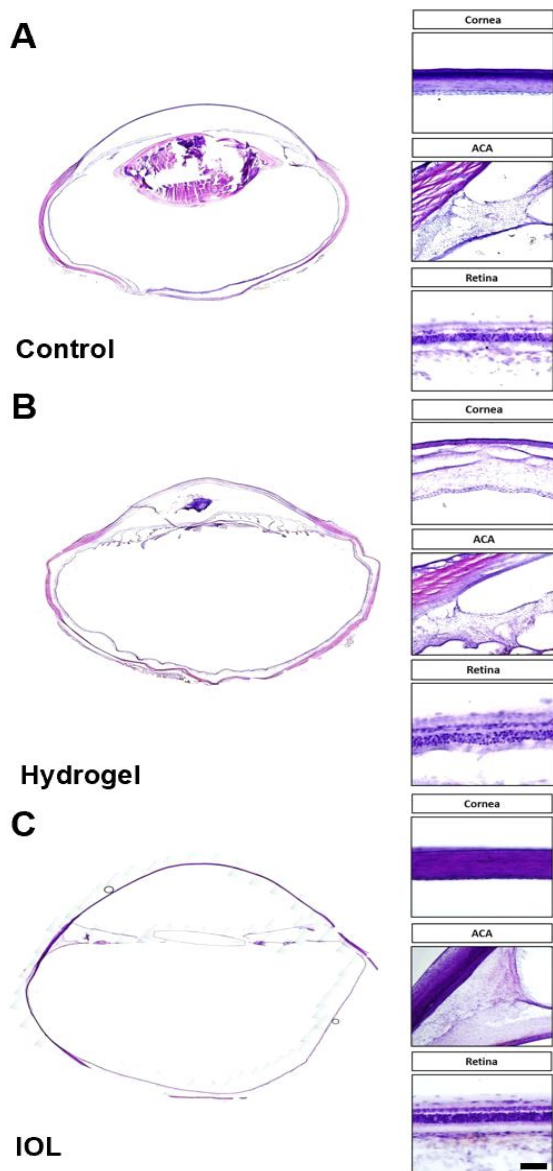


Figure 6 The poly(acrylamide-co-sodium acrylate) hydrogel does not cause histological alteration 3 months post-implantation

A: HE staining of New Zealand White Rabbit eyes showed the detailed histological identity of the cornea, anterior chamber angle, and retina in the control group; B: HE staining of New Zealand White Rabbit eyes showed the detailed histological identity of the cornea, anterior chamber angle, and retina in the hydrogel group. The cell connectivity and layered lamination were normal compared with those of the control group, even though the cornea was ruptured due to the limitations of the HE staining procedure; C: HE staining of New Zealand White Rabbit eyes showed the detailed histological identity of the cornea, anterior chamber angle, and retina in the IOL group. Scale bar, 100 μm .

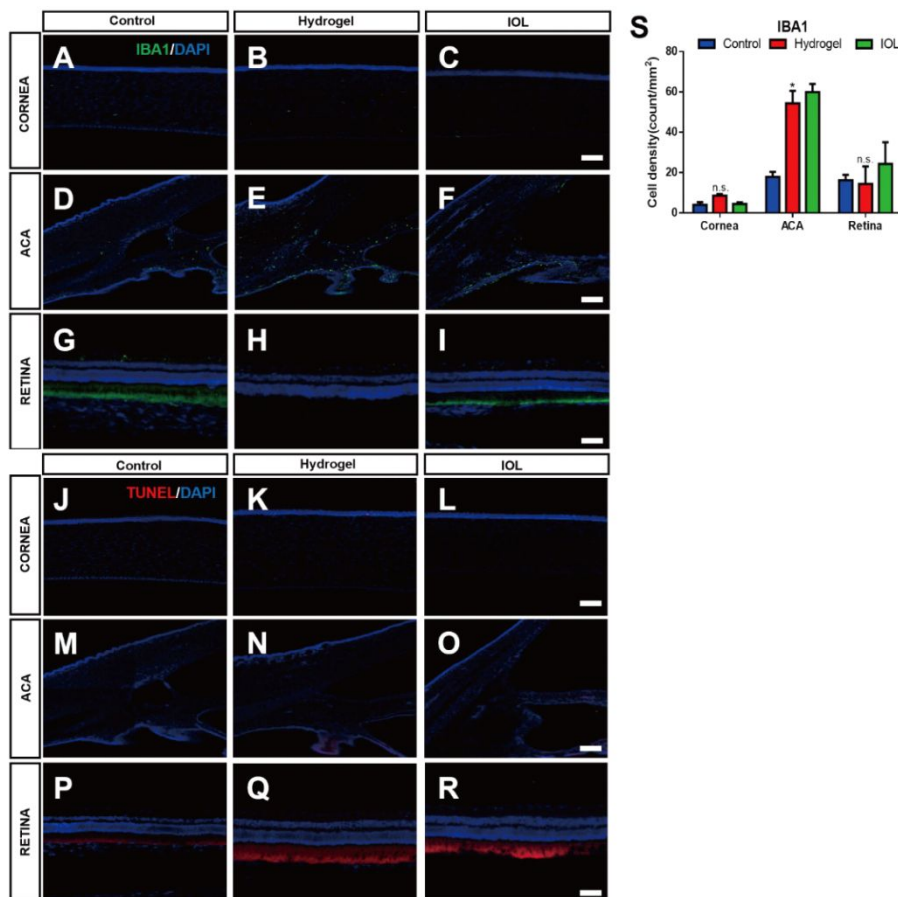


Figure 7 Macrophage activation and cell apoptosis of New Zealand white rabbit eyes 3 months after poly(acrylamide-co-sodium acrylate) hydrogel implantation

A~I: Immunostaining of Iba1 in New Zealand white rabbit eyes at 3 months post-implantation showed Iba1+ macrophage augmentation at the cornea (A~C), ACA (D~F) and retina (G~I) in the control group (A, D, G), the hydrogel group (G, E, H), and the IOL group (C,F,I); J~R: TUNEL assay of New Zealand White Rabbit eyes at 3 months post-implantation showed TUNEL+ apoptotic cell accumulation at the cornea (J~L), ACA (M~O) and retina (P~R) in the control group (J, M, P), the hydrogel group (K, N, Q), and the IOL group (L, O, R); S: Statistical analysis of Iba+ macrophages in the cornea, ANA, and retina in all three groups. Scale bar, 100 μ m. Data are shown as the mean \pm s.d. Independent sample t test. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.005$; n.s. no significance.

DISCUSSION

The eye exerts a unique ocular morphology, and all ocular organic architectures are susceptible to a number of diseases that may require treatment via different modalities, such as sustained drug delivery and artificial biotissue substitution.¹⁸ In the past century, hydrogels have been used in various applications as effective materials. The unique network structure of a hydrogel makes it highly hydrophilic and biocompatible, and it exhibits soft physical properties similarly to living tissue, which makes it an ideal and potential biomaterial for ophthalmic applications such as intraocular pumps, injections and implants, reducing comorbidities caused by glaucoma, cataracts, and diabetic retinopathies.¹⁹ 3D printing allows the creation of objects with complex three-dimensional geometries and photologies from a computer-aided design and printed biomaterials to tissue analog structures without any change in their mechanical or biological properties.^{20,21} However, given the nature of the polymers in hydrogel formulations and the materials used in the preparation of ophthalmic gels, it is paramount that any new hydrogel formulation intended for ocular application should be inevitably investigated for potential toxicity/adverse effects on ocular tissues. In this report, we generated a poly(acrylamide-co-sodium acrylate) hydrogel as a thin film and found that the newly formed hydrogel needs 9 days of pretreatment with PBS to stabilize its structure, reflecting the stimuli-controlled phase transition and small molecule release characteristics of stimulus-responsive hydrogels²². Second, we performed coculture experiments *in vitro* and implantation experiments *in vivo* to assess the effect of the hydrogel on ocular tissue. The biosafety research parameters for structure, function, and inflammation, such as cell proliferation assays, inflammation detection by slit lamp and aqueous humor tests, visual function assay by electroretinogram, and anatomical abnormality detection by HE staining and immunostaining, all indicated that the poly(acrylamide-co-sodium acrylate) hydrogel is qualified as a biosafe clinical-grade biomaterial for 3D-printed IOLs by systematic investigation and verification both *in vivo*

1
2 and *in vitro*.

3 The polymers in preformed ophthalmic hydrogels could be semisynthetic or natural. Some of them can transit
4 from sol to gel conversely triggered by environmental stimuli such as temperature, pH and ion concentration^{11,23}.
5 As the poly(acrylamide-co-sodium acrylate) hydrogel was primarily performed by 3D printing, it was harmful to
6 cell proliferation when co-cultured with LECs. (Figure 1C, D). However, pretreatment of the
7 poly(acrylamide-co-sodium acrylate) hydrogel with PBS for 9 days robustly reduced the toxic effect to LECs.
8 This result indicated two points of view: first, the primary poly(acrylamide-co-sodium acrylate) hydrogel could
9 respond to the cell culture environment conditions and release polymers that inhibit cellular proliferation at a high
10 concentration; second, adaptation to the extracellular environment is needed and should be properly investigated
11 and standardized for the safe clinical use of poly(acrylamide-co-sodium acrylate) hydrogels.

12 When co-cultured with the poly(acrylamide-co-sodium acrylate) hydrogel, both LECs and ARPE19 cells
13 exhibit contact cell death, while cells located far away from the hydrogel grow without any abnormalities. Cell
14 migration and proliferation are highly dependent on the extracellular matrix²⁴. To serve as an ideal environment
15 and matrix, ophthalmic hydrogels must be composed of gelatin²⁵, alginate²⁶, collagen²⁷, fibrin²⁸, and even
16 molecularly modified biomaterials. 3D-printed IOLs have a high light transparency but may be populated by
17 migratory and proliferative cells^{29,30}. The contact cell death of the poly(acrylamide-co-sodium acrylate) hydrogel
18 prevented this possibility of this adverse effect of cell migration and proliferation, which makes it an ideal
19 transparent biomaterial with the least opacity after IOL implantation.

20 A previous study on the ocular tolerability of a new biomaterial formulation used the “Draize rabbit eye
21 irritation test,” which is the oldest and most classic test that has been employed to evaluate potential ocular
22 irritation. However, the Draize test is quite limited by its subjectivity, poor reproducibility and the need for large
23 numbers of live rabbits^{31,32}. In the current research, we utilized ELISA to explore the inflammatory factor level of
24 the aqueous humor, and we conducted electroretinogram analyses to examine the visual function after hydrogel
25 implantation. These analyses well addressed the shortcomings of the Draize test and provided distinguished data
26 to gain insight into the effect on ocular inflammation and visual function.

27 Macrophages are a type of immune cell that engulfs and digests debris, foreign substances, and microbes³³.
28 They are patrol guards in the microenvironment of all tissues, which are transformed into active phagocytes by
29 morphology and function and react quickly to various kinds of tissue damage. There is conclusive evidence in
30 animal models and in situ analysis of human tissues that the macrophage response is a common feature of various
31 retinal inflammatory diseases³⁴⁻³⁸. We found accumulated Iba⁺ macrophages in the anterior chamber angle and iris
32 of both hydrogel groups 3 months post-operation. The morphology of Iba⁺ macrophages in the anterior chamber
33 angle and iris is amoeboid like, suggesting that the phagocytic amoeboid movement of emerging macrophages is
34 triggered by hydrogel implantation. Most importantly, we found similar results in the IOL group, which served as
35 a positive biosafe control group, and no corneal opacity, keratic precipitate, hypopyon, or synechia were observed
36 in the hydrogel or IOL group.

37 As the implanted IOL will be left in the eye for several years after surgery, there is growing interest in the use
38 of IOLs as drug reservoirs or as treatment methods for ophthalmic diseases. Combining 3D printing technology
39 and the biocompatible poly(acrylamide-co-sodium acrylate) hydrogel as the printing material, as well as a
40 potential material for stimuli-responsive hydrogel fabrication, we hold the possibility of loading antibiotics,
41 corticosteroids, and NSAIDs in the IOL, which integrates cataract surgery and postoperative treatments as a
42 one-step procedure³⁹. Moreover, IOLs can also be incorporated with telescopic lenses, presbyopia-correcting
43 lenses, or accommodative polyfocal bioanalogical lenses^{40,41}. These advanced designs could become more
44 effective and personalized when combined with 3D printing and biocompatible hydrogels. However, all these
45 IOLs inevitably require rigid biosafety assessments and more clinical studies to ascertain their safety and
46 effectiveness.

47 In sum, the implantation of the poly(acrylamide-co-sodium acrylate) hydrogel did not disturb the IOP, blood
48 parameters, electroretinogram or optical structure, indicating that it will not cause any ocular irritation. However,
49 it is not clear whether this hydrogel lens maintains the nano smooth surface, which can hopefully to correct visual
50 errors, including the defocusing and astigmatism caused by traditional IOLs. We will improve the manufacturing
51 process in the future and test the correction capacity of this new lens in terms of visual quality. Our research
52 provides insight into the biosafety of poly(acrylamide-co-sodium acrylate) hydrogels in ocular tissue and
53 demonstrates that poly(acrylamide-co-sodium acrylate) hydrogels are a safe material for 3D printing of personal
54 IOLs, which hold great potential for future clinical applications.

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