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Poly(2-ethyl-2-oxazoline) grafted gellan gum for potential application in transmucosal drug delivery

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Gellan gum (GG) has been used to prepare polymeric carriers with prolonged retention on the eye surface for topical ocular drug delivery. In this work, GG was chemically modified with short poly(2-ethyl-2-oxazoline) (PEtOx) chains that were expected to have minimal adhesion to mucosal tissues (mucoadhesion). The choice of synthetic procedure, solvents, and reagents has been dictated by biocompatibility of the materials and possible application in drug delivery. The grafts were synthesized via cationic ring-opening polymerization and their living chains were attached onto deprotonated gellan backbone. The derivatives with three degrees of grafting were prepared by varying the in-feed mass ratio of PEtOx grafts over GG. NMR and FT-IR spectroscopies, thermogravimetric analysis, and SEC evidenced that the grafting had actually taken place. However, a greater diffusion coefficient determined for the copolymer, using diffusion-ordered spectroscopy (NMR), in relation to the diffusion of the unmodified GG, suggested either partial degradation of the backbone or a more compact structure of the copolymer. GG and its graft copolymers (GG-g-PEtOx) were found to be highly biocompatible with cells cultured under their induction at concentration of 1, 0.1 and 0.01 mg/mL demonstrated a physiological morphology, as well as an increase in viability and proliferation.

KEYWORDS
gellan gum, graft copolymer, mucoadhesion, mucus penetration, poly(2-ethyl-2-oxazoline)

1 | INTRODUCTION

Ocular drug delivery is a challenging field of pharmaceutics owing to specific conditions on the eye surface. Many drug carriers often have disadvantages such as poor retention on ocular surfaces; additionally their ocular administration may cause irritation and blurred vision. Topically administered drugs are easily diluted by tears or removed by blinking, which results in subtherapeutic drug levels.[1] Ongoing demands exist for innovative drug carriers capable of prolonged retention on the ocular surface and enhanced effectiveness of therapy. Gellan gum (GG) is an attractive material that has already found applications in the design of dosage forms for ocular drug delivery due to its mucoadhesive properties and ability to form gels in situ.[2]

GG is an anionic tetra-saccharide with a repeating sequence comprising of D-glucuronate, L-rhamnose and two D-glucose units.[3] GG is typically produced in a fermentation process by *Pseudomonas* bacteria isolated from *Elodea* plant.[4] The native form of GG contains on average one glyceryl and 0.5 acetyl groups per glucose unit that can be

removed by hot alkaline treatment giving the deacetylated form, so called low-acyl GG.[5] Both native and low-acyl GG form gels. Gels formed by the native GG are typically elastic and weak, whereas more compactly packed low acyl GG produces hard and brittle gels. Gelation of GG occurs upon cooling and the gel turns back to fluid if heated again.[6] Gelation is also promoted by minute amounts of mono and divalent metal cations, which is not always the case with all gelling polysaccharides.[7-8] The advantages of GG over the other gelling polysaccharides are the thermal and enzymatic stability and high strength of the gel.

GG has been approved as a gelling agent in foods (E418, FDA code 21CFR172.665).[9] Its low toxicity, biodegradability, commercial availability, and low cost render GG suitable for applications in drug delivery and tissue engineering.[10-12]

Tear fluid contains over four times excess of cations required to promote gelling of a 1 wt% GG solution.[13] Therefore, effective ophthalmic drug delivery can be thought in two phases: liquid formulation facilitates the administration of the drug onto the eye surface, whereas subsequent gelation promoted by tear fluid resists ocular drainage and prolongs the drug retention time. Thus, significant increase in the contact time due to the fast ion-activated in situ gelling has been demonstrated for the low acyl GG.[14] Administration of solutions on human and rabbit eyes showed minimal irritation, though some blurred vision was observed.[15]

Above-mentioned applications employ mucoadhesive properties of GG. It has recently been demonstrated that modification of silica nanoparticles surface with poly(2-methyl-2-oxazoline) and poly (2-ethyl-2-oxazoline) (PEtOx) decreases mucoadhesion and facilitates wthe diffusion of the particles in gastric mucin,[16-17] whereas more hydrophobic poly(2-*n*-propyl-2-oxazoline) shows no significant increase in the particles mobility compared to the pristine particles.[17] As a development of this research, we propose the modification of GG with short PEtOx chains.

PEtOx is chemically stable, non-toxic, and has structural similarities with polypeptides owing to the amide group in its repeating units. It has shown stealth properties comparable with poly(ethylene glycol), that is, low recognition by the immune system.[18-22] These features have risen interest in PEtOx for biomedical applications.[23-27] Homogeneous and controlled modification of GG with PEtOx is not an easy task owing to the gelling of GG and the limited number of common solvents appropriate for PEtOx synthesis and modification. Additionally, the chemicals and synthetic reactions used ought to be biologically safe, which rules out click-reactions for well-defined grafting. Post-functionalization of intentionally terminated end-groups of PEtOx has to be also avoided.

Therefore, in this research, GG was modified by grafting with short PEtOx chains to prepare graft copolymers of various degrees of grafting. The length of the PEtOx blocks was selected to be approximately 5000 g/mol, which has been shown to have mucus penetrating properties.[16,17]

PEtOx has been synthesized via cationic ring-opening polymerization (CROP).[28] The method suggested for poly(2-isopropyl-2-oxazoline-*co*-2-butyl-2-oxazoline) grafting to β-glucan[29] and to κ-carrageenan[30] was adjusted to GG-g-PEtOx. A key factor for successive PEtOx synthesis followed by its grafting to deprotonated GG is the living nature of polymerization with a minimum number of chain transfer and termination reactions. The living chain ends can be terminated with nucleophilic reagents. Therefore, special care has been taken of purity and dryness of chemicals. Three GG-g-PEtOx samples with different degrees of grafting were synthesized.

2 | EXPERIMENTAL

2.1 | Materials

Chemicals were purchased from Aldrich and used as received unless stated otherwise. Calcium hydride (CaH_2, 95%) was used for drying 2-ethyl-2-oxazoline (EtOx, ≥99 %) and sulfolane (99%). Methyl trifluoromethanesulfonate (MeOTf, 98%) and methyl *p*-toluenesulfonate (MeOTs, 97%) were used to initiate polymerization. Dimethyl sulfoxide (DMSO, 99.99%, Fischer Scientific Oy) and toluene (≥99.9 %, Merck Millipore) were dried over molecular sieves (3 Å, VWR) prior to use. Sodium hydride (NaH, 60% dispersion in mineral oil), diethyl ether (anhydrous, J.T. Baker) were used in the synthesis too. Tetrahydrofuran (THF, ≥99.9 %, Honeywell) was used for drying GG. Low acyl GG was purchased from Zhejiang DSM Zhongken Biotechnology Co., Ltd. (M_n = 10^6 g/mol estimated by viscosity[31]). Acetone (≥99.8 %) and isopropanol (≥99.5 %, Fischer-Chemicals Oy) were used in the purification of GG. Deuterated chloroform ($CDCl_3$, 98% D + 0.03% Tetramethylsilane, Euriso-Top) and deuterated water (D_2O, 99% D, Euriso-Top) were used in NMR experiments. Dialysis membranes (molecular weight cut off 3.5 kDa and 12-14 kDa) were purchased from Spectrum laboratories. Milli-Q water, obtained from a Millipore apparatus, with a resistivity of 18.2 MΩ cm at 298 K was used for all experiments.

2.2 | Selection of solvent

Preparation of GG-g-PEtOx proceeds in two main phases: polymerization of PEtOx via CROP and then attachment of living PEtOx chains to deprotonated GG at 70°C. An appropriate solvent, which is common for GG and PEtOx and also suitable for CROP is a prerequisite for this synthesis. Commercial low acyl GG is poorly soluble in other solvent than water, whereas PEtOx synthesis cannot be conducted in water. Acetonitrile is a typical solvent for CROP,[32] however GG is not soluble in it. Water-free DMSO do not cause significant gelling of gellan[33] and expected to be good candidate for this synthesis. DMSO and sulfolane are aprotic dipolar solvents that can be used for CROP.[34] Sulfolane is less hydroscopic than DMSO. Sulfolane is slightly more toxic than DMSO, while chain transfer is more probable in DMSO.[32] Commercial GG is not completely soluble neither in sulfolane nor in DMSO in the "as received" form, though it swells in sulfolane. Therefore, GG was purified as described below. After purification, GG becomes soluble in DMSO, which was selected as the solvent for grafting. The polymerization of EtOx was performed in

sulfolane and then the solution of living PEtOx chains was added to the DMSO solution of GG.

2.3 | Preparation of GG for modification

Commercial low acyl GG is in a powder form and per se cannot be homogeneously modified. A 1 w/v % GG solution is not uniform even in water; it forms a fraction of soluble chains and a dispersed fraction of swollen gellan particles. GG may contain Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}, from which divalent cations impair the solubility the most and act as intermolecular crosslinks.[35] Proteins remaining after fermentation are another possible source of impurities.[4] However, elemental analysis did not show contamination of GG with proteins (see Supporting Information).

The network-like particles were removed in two consecutive precipitations. At first, gellan (1 w/v %) was dissolved for 3 h in deionized water at 50°C. Turbid solution/dispersion (see Supporting Information) was centrifuged for 30 min at 40°C with a spinning rate of 5000 rpm. The clear supernatant fraction was collected and precipitated in acetone (1:4 v/v). GG was separated from water-acetone mixture by vacuum filtration using Whatman Grade 541 filter paper and dried for ~30 min. Then, the separated GG was redissolved in deionized water again, reprecipitated in acetone and dried. Dry GG was twice washed with isopropanol. Then, GG was dissolved in deionized water and dialyzed against deionized water (cut off 12–14 kDa) for at least 24 h. The product was freeze-dried and collected as dry fluffy white fibers.

The yield of purification was 45%–50% of the original weight of GG. FT-IR and NMR spectroscopy did not reveal any significant differences between purified and pristine GG. Thus, there was no indication that GG was incompletely deacetylated and that a fraction was removed during purification. This conclusion is also supported by the elemental analysis, which reveals equal carbon and hydrogen contents in both samples. Therefore, the most likely reason for the low yield of purification is divalent cations. Small amount of these metal cations binds gellan chains into particles, which are removed by centrifugation.

Purified GG homogeneously dissolves in water and becomes soluble in DMSO. However, it is not soluble in sulfolane but remains soluble in DMSO/sulfolane mixtures used in this report. This determined the choice of DMSO as the solvent for grafting.

Freeze-dried GG is highly hydroscopic. Azeotropic distillation with tetrahydrofuran and toluene was performed prior to grafting to remove absorbed moisture. Two azeotropic distillations were tested for the GG drying. In the first, GG was dried during distillation of THF and was stored in a vacuum desiccator over silica gel. Thermogravimetric analysis and NMR spectroscopy estimate the water content of dried GG to be about 6%. The other method was shown to be more effective. GG (200 mg, 1.14 mmol that is an averaging of the four sugar units) was first dissolved in 20 mL of anhydrous DMSO. The solution was dried by addition of anhydrous toluene (3 × 5 mL) and subsequent evaporation under reduced pressure. If several reactions were performed simultaneously, excess GG solution was prepared, which was at this stage split into separate flasks.

2.4 | Synthesis of PEtOx

Glassware used in the synthesis and distillations was kept overnight in an oven at 90°C prior to use. Calcium hydride was used for drying EtOx and sulfolane. EtOx placed in a round-bottom flask, and CaH_2 was added until the mixture turned milky. Drying tube filled with calcium chloride granules was connected to the flask and the mixture was stirred until the next day. For sulfolane, drying was identical, but the flask was placed in an oil bath set to 32°C, which was above the freezing point of sulfolane (28.4°C).[36] Next day EtOx was distilled under a nitrogen atmosphere and sulfolane under reduced pressure.

Polymer synthesis described in Loukotová et al.[29] was adapted. The monomer-to-initiator ratio was 50:1 in the PEtOx polymerizations targeting the molar mass of 5000 g/mol. The trial polymerizations were performed using methyl *p*-toluenesulfonate (MeOTs) as an initiator. Although these polymerizations proceeded successfully, the solid form of the initiator at room temperature made handling challenging and for that reason MeOTs was replaced with methyl trifluoromethanesulfonate (MeOTf). MeOTf was extremely reactive as initiator and could not be added directly to the hot reaction mixture: loss of the initiator was observed due to the evaporation at elevated temperature and subsequent condensation on the reaction flask walls. The addition of MeOTf to monomer mixtures has been reported at 0°C[37] and at room temperature[18] followed by heating. Because sulfolane is solid under 28°C, the handling of the initiator at lower temperatures was not possible.

For the synthesis of living PEtOx grafts, the polymerization procedure started by bubbling EtOx (2 g, 20.18 mmol) and sulfolane (4 mL) with nitrogen at 35°C for 30 min. Higher monomer concentration results in too viscous polymer solution, which is not practical. About 100 mg/mL solution of MeOTf was prepared in another flask (5 mL) and purged for 15 min with nitrogen at 35°C. The solution of the initiator (0.66 mL) was transferred to the reaction mixture with a needle. The mixture was stirred at a moderate stirring rate for 60 min and then the temperature of the oil bath was raised to 70°C. The stirring of the mixture was continued for about 24 h under a nitrogen atmosphere. Further details of PEtOx polymerization and analysis of the PEtOx homopolymers (samples P1–P5) are presented in Supporting Information. PEtOx sample P5 (M_n = 7960 g/mol) was used in the synthesis of the GG-g-PEtOx copolymers.

2.5 | Synthesis of GG-g-PEtOx

Procedure for the GG-g-PEtOx synthesis is shown in Figure S5. Gellan chains were grafted in two parallel processes starting with polymerization of EtOx. The GG backbone was activated with NaH, producing alkoxide ions. However, grafting can also take place at carboxylic groups of D-glucuronate. In the course of the grafting, a new covalent ether bond is formed between a PEtOx chain and GG.

Grafting was tested at three temperatures. Grafting at 70°C resulted in extensive degradation of gellan gum. Synthesis at and below 40°C promotes gelling that is not favorable. About 50°C was selected for the reported polymers.

Sodium hydride (340 mg, 8.4 mmol) was added to the 10 mg/mL solution of GG in DMSO azeotropically dried using toluene (200 mg, 1.14 mmol that is an averaging of the four sugar units was dissolved in 20 mL). The mixture was stirred for 3 h at 50°C. A needle was placed through the rubber septum to release formed hydrogen gas. Next, the pre-determined amount of living PEtOx chains in sulfolane (sample P5) was added with a needle to the gellan/DMSO mixture, and the mixture was stirred overnight. Polymerization of the remaining PEtOx chains was terminated by the addition of 1 M methanolic NaOH and this mixture was stirred at room temperature for 24 h to confirm complete termination. Finally, the PEtOx mixture was dialyzed (cut off 3.5 kDa) for a week, freeze-dried, and characterized.

Fifteen milliliters of water was added to the resulted mixture and the mixture was then poured into the 250 mL separatory funnel. The mineral oil was removed by washing the mixture with 50 mL of diethyl ether and separating the aqueous phase. This was done at least twice. If gelling occurred during this washing, warm water was added to the separatory funnel and the mixture was shaken to dissolve the gel. Finally, the aqueous mixture of GG-g-PEtOx with unreacted PEtOx was dialyzed (cut off 12–14 kDa) for a week to remove PEtOx and other low molar mass impurities. The product was isolated as white fibers after freeze-drying.

2.6 | Characterization

Elemental analysis was used to determine the composition of the commercial GG as received in comparison to purified GG. Elemental composition was determined by the HANAU Elementar Analysensysteme GmbH, Germany.vario MICRO cube using sulfanilamide as a standard. Three samples (~2 mg of dry polymer per measurement) were studied and the average of those values is presented as a result.

NMR spectroscopy: Zg20 pulse sequence was applied to record ^{1}H NMR spectra with a Bruker Avance III 500 spectrometer. In diffusion-ordered spectroscopy (DOSY) experiments, ledbpgp2s pulse sequence was used: longitudinal eddy current delay (LED) with bipolar gradient pulse pair, two spoil gradients. Parameters used in DOSY: 32 scans, diffusion time d20 was set to 0.1 s, gradient pulse 2%–95%, and delay d1 was 5 s. The spectra were recorded at room temperature in D_2O (10 mg/mL concentration for solutions of GG-g-PEtOx and PEtOx). The concentration of pristine gellan was 3–5 mg/mL. The conversion samples of PEtOx were recorded in $CDCl_3$ and contained a couple of drops of polymerization mixture. Bruker's TopSpin™ 3.6.2 software was used for analysis of spectra.

Size exclusion chromatography (SEC): Molar mass analysis was performed in dimethylformamide (DMF) at a flow rate of 0.8 mL/min. The SEC setup consisted of Waters 515 HPLC pump, Waters 2410 RI detector, and Waters Styragel HR2, 4, and 5 columns. The temperature of the columns was 30°C. Samples were calibrated against poly(methyl methacrylate) (PMMA) standards (Polymer Standard Service) and analyzed with OmniSEC software.

Fourier Transform Infrared (FT-IR) spectra were obtained by means of a PerkinElmer Spectrum One FT-IR spectrometer within the scanning range of 650–4000 cm^{-1}. Attenuated total reflection sampling was used. The spectra were plotted and analyzed using the Origin 2018 software.

Thermogravimetric analysis (TGA) was performed using a Perkin Elmer TGA 850 instrument. Samples (3–5 mg) were heated from 25 to 800°C with a heating rate of 10°C/min in a nitrogen atmosphere. Thermograms were plotted and analyzed using the Origin 2018 software.

Dynamic light scattering (DLS) measurements were performed using a Zetasizer NanoZS instrument (Malvern Instruments Ltd.) equipped with a 4 mW He-Ne laser operating at λ = 632.8 nm. The scattered light intensity from 0.05 wt% aqueous solution of the graft copolymers was collected at a scattering angle of 90° at 25°C. All solutions were filtered through 0.45 μm filters. The reported values are the intensity-weighted diameters, which were calculated as averages of three repetitive measurements.

Scanning electron microscopy (SEM) images were obtained using a field emission scanning electron microscopy (FESEM; JEOL JSM 7000F) at an accelerating voltage of 15 kV. The samples for SEM were prepared by depositing a droplet of a 0.05 wt% aqueous solution of the graft copolymers on a silicon substrate and allowing it to dry overnight at room temperature. The sample was sputter coated with 10 nm thick Au to minimize charging, before the measurement.

The optical transmittance of the graft copolymers aqueous solution (0.7 w/v %) was measured at 650 nm using a Lambda 25 PerkinElmer UV/vis spectrophotometer as the solution temperature was raised from 20 to 80°C.

2.7 | In vitro biocompatibility assessment

Cell culture maintenance: The fibroblast-like cell line L929 (DSMZ Braunschweig, Germany, ACC-2) established from normal subcutaneous areolar and adipose tissue of a male C3H/An mouse was used for the cell viability and proliferation assessment. This is a relevant cell type for the biocompatibility testing of materials. Cells were cultured in RPMI culture medium (biosera), supplemented with 10% Fetal Bovine Serum (FBS) 10% v/v (Gibco), 50 IU/mL penicillin (Sigma-Aldrich, St. Louis, MO), 50 g/mL streptomycin (Sigma-Aldrich, St. Louis, MO) in a 5% CO_2 incubator (Heal Force) at 37°C.

Cell viability and proliferation assay: Cell viability was evaluated according to ISO 10993-5 (2009) standards and measured by the PrestoBlue® viability assay (Invitrogen) according to our protocol[38] and the manufacturer instructions. For the assessment of gellan gum (GG) and its graft copolymers (GG-g-PEtOx), 5×10^3 cells per well were seeded in 96 well plates, and after 24 h, the culture medium was replaced with new medium containing the samples at the concentrations of 1, 0.1, and 0.01 mg/mL. Cell viability was assessed on days 2, 4, and 7 by measuring the absorbance at 570 and 600 nm in a spectrophotometer (Synergy HTX Multi-Mode Microplate Reader, BioTek, Bad Friedrichshall, Germany). After the measurement at each time point, new medium containing the samples was added to the cells.

Tissue culture treated polystyrene (TCPS) was used as a control surface for the cells.

Cell morphology by optical microscopy: The cells placed in multiwell plates and cultured under the induction of GG and GG-g-PEtOx, were examined daily for 7 days and visualized by means of a Zeiss Axiovert 200 optical microscope. Images were taken from the cells containing the highest concentration of 1 mg/mL of the materials by a ProgResVR CFscan Jenoptik camera (Jena, Germany) using ProgResVR CapturePro 2.0 software and objective lenses for 10-fold magnification.

Statistical analysis was performed for the cell viability assessment using the one-way ANOVA Tukey's multicomparison test ($n = 6$) in GraphPad Prism v.8 software. At each time point, the values of the materials of one concentration were compared to those of the other two concentrations. A probability value of $p < 0.01$ was considered insignificant, if no other indication is stated.

3 | RESULTS AND DISCUSSION

3.1 | Characterization of GG-g-PEtOx

GG-g-PEtOx copolymers of three different grafting densities were prepared by adjusting the living PEtOx content in the reaction feed (Table 1). This is given as weight equivalent in feed, that is, the mass ratio of PEtOx grafts over GG used in the grafting reaction. The mass content of PEtOx is estimated from the assumption that all the initiator reacts and forms a propagating chain. This may deviate from the actual molar amount as M_n of PEtOx was higher than the theoretical molecular weight. Also, the molar mass of the attached PEtOx grafts may slightly differ from the M_n value measured for the corresponding PEtOx sample P5 obtained by polymerization termination.

Figure 1 shows ^{1}H NMR spectra of pure gellan gum and the GG-g-PEtOx copolymers. The rhamnose unit is represented with two separate peaks: at 5.03 ppm for the CH proton and a relatively intensive peak at 1.18 ppm for the methyl protons. The peak at 4.43 ppm corresponds to two CH protons of the first glucose unit and one CH proton of the rhamnose unit.[3]

^{1}H NMR spectra of the G1–G3 graft copolymers show typical peaks of GG: methyl protons of rhamnose unit at 1.18 ppm and CH protons on both sides of the solvent peak. The intensity of these signals decreases as the PEtOx content increases indicating a higher degree of grafting. The characteristic peaks of PEtOx are also present at 0.97 and 2.26 ppm for CH_2 and CH_3 protons of the ethyl side group and at 3.44 ppm for the backbone. The degree of grafting was estimated using the peaks from the methyl end group (2.84–2.98 ppm) corresponding to PEtOx.

The grafting density is given as the number of repeating sequences (i.e., four sugar units constituting GG) per one PEtOx graft. Hence, every 12th repeating sequences (i.e., 12 × 4 sugar units) of the G1 copolymer has in average one PEtOx grafted chain. In the case of G2, the grafting density is 3, and for G3, that is 2.

The peak intensity from the end-group is low and the peak from rhamnose becomes evident at the higher GG contents and overlaps with another peak at 0.96 (methyl protons of the side group of PEtOx), the accuracy of the grafting density calculation is not perfect. However, the difference in grafting densities between the copolymers is evident. Examination of the yields of the gellan copolymers indicates increasing graft content relative to the weight equivalent.

Figure 2 shows FT-IR spectra of the GG-g-PEtOx copolymers. Spectra of pure GG and PEtOx homopolymer are given in Figures S6 and S7. The band at 3447 cm^{-1} corresponds to GG and is practically the same for all copolymers. The varying intensity of the peaks at 1632 and 1422 cm^{-1} proves the presence of PEtOx and shows a decreasing trend with decreasing the degree of grafting. The peak at 1052 cm^{-1} may be a superposition of two contributions: one comes from GG (1039 cm^{-1}) and corresponds to the stretching of the C—O bond of the hydroxyl group, whereas the other one comes from PEtOx (1061 cm^{-1}) and corresponds to the tertiary amide. The intensity of this peak does not change with degree of grafting because of the equivalent formation of new CH—O—CH_2 bonds between PEtOx and GG.

Thermograms of pure GG, PEtOx, and the GG-g-PEtOx copolymers are presented in Figure 3. GG shows a two-step degradation process. Six percentage weight loss of absorbed water is observed in the temperature range of 25–100°C. Decomposition of the GG backbone takes place between 200 and 300°C (about 50% of the weight). The fastest weight loss occurs at 247°C (Figure S8). At 800°C, the total weight loss was 76%. Degradation of PEtOx happens between

TABLE 1 Experimental conditions for the GG-g-PEtOx copolymer synthesis

Sample	*m* (gellan) (mg)	*n* (living EtOx)[a] (mmol)	*m* (PEtOx)[b] (mg)	*w*, equiv.[c]	Yield (mg)	Repeating sequences per grafted PEtOx[d]
G1	216	0.045	360	2	284	12
G2	212	0.135	1076	5	524	3
G3	206	0.306	2433	12	749	2

Note: In this synthesis, M_n of the PEtOx grafts was 7960 g/mol by ^{1}H NMR (sample P5 in Supporting Information).

[a]An estimation from the sample weight of living PEtOx in relation to the total mass of PEtOx mixture.

[b]m(PEtOx) = n(living PEtOx) × M(PEtOx).

[c]m(PEtOx)/m(gellan gum).

[d]Estimated by ^{1}H NMR. Repeating sequence is a block of four sugar units constituting GG.

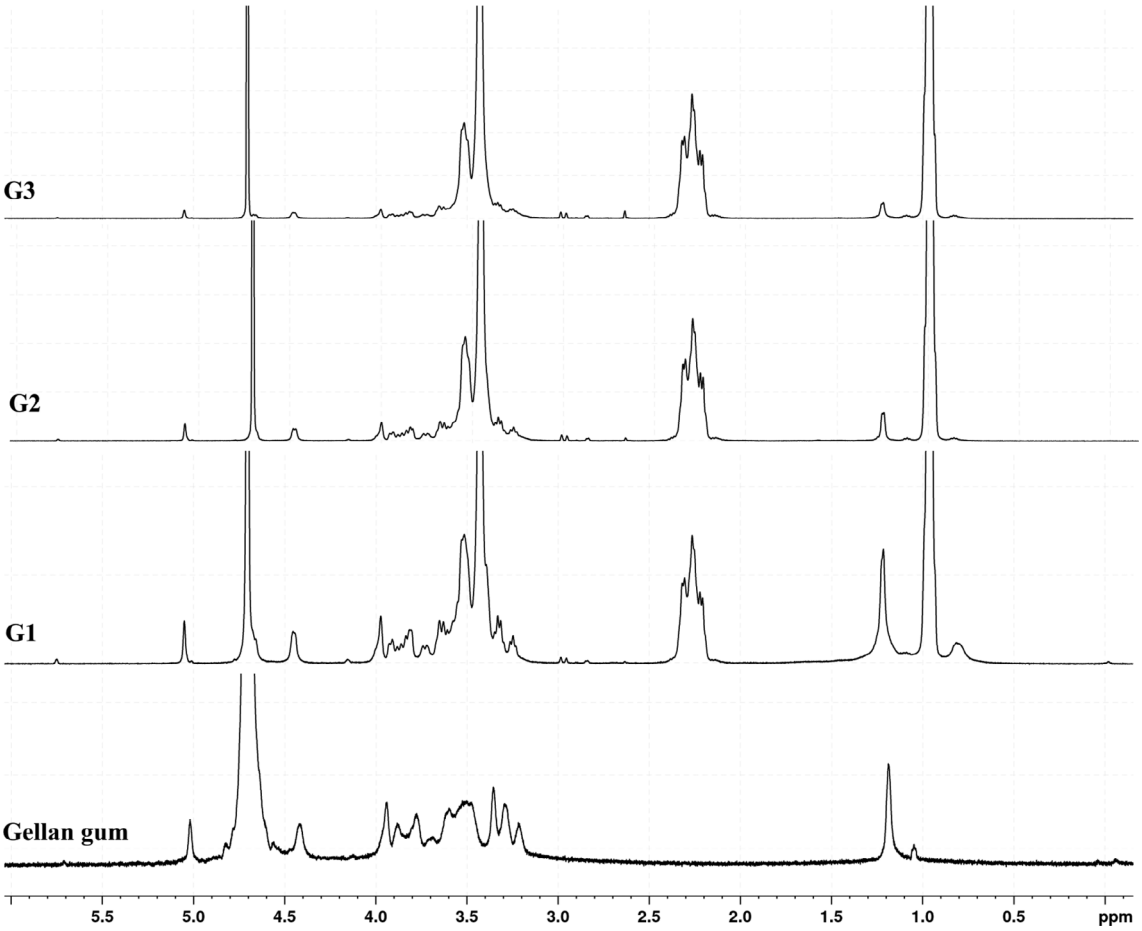

FIGURE 1 ^{1}H NMR spectra of pure gellan gum and the GG-g-PEtOx copolymers. Assignments of the peaks to corresponding chemical structures are given is Figures S2 and S3

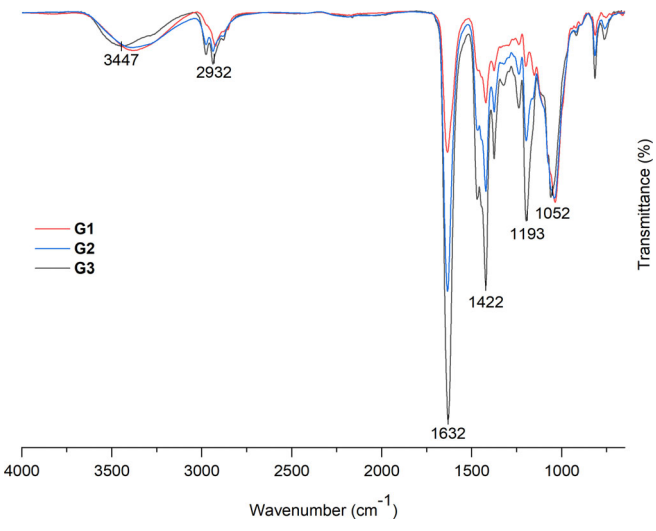

FIGURE 2 FT-IR spectra of the GG-g-PEtOx copolymers. FT-IR spectra of gellan gum and of the PEtOx homopolymer, sample P5 and the bands assignments are given in Figures S6 and S7 correspondingly

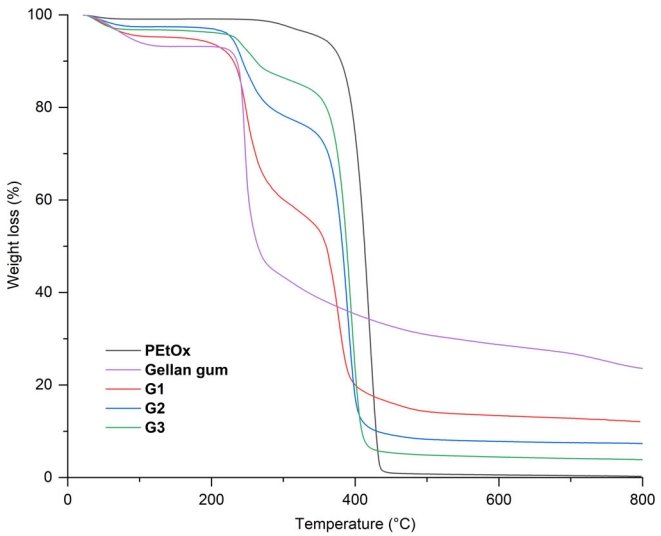

FIGURE 3 Thermograms of pristine gellan gum, PEtOx homopolymer and the graft copolymers

260 and 440°C. The fastest degradation appears at 420°C (Figure S8). The PEtOx decomposition proceeds to almost 100% weight loss and results in negligible char yield.

All thermograms of the GG-g-PEtOx copolymers show weight loss within 25 to 100°C, indicating the presence of water. The higher moisture content corresponds to the copolymer with the higher GG

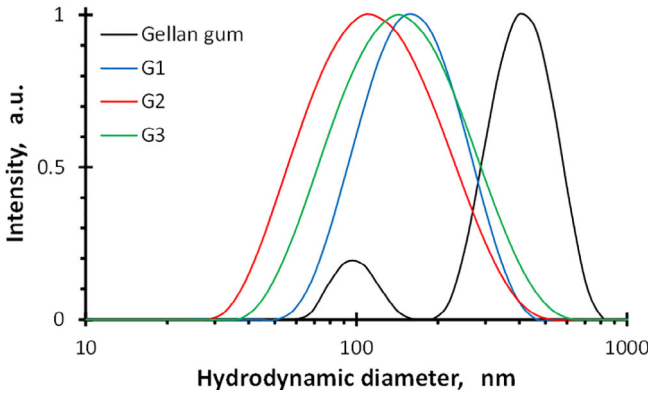

FIGURE 4 Distributions of the hydrodynamic diameter of gellan gum and its graft copolymers. Measurements are performed at 25°C using aqueous 0.05 wt% solutions at 90° scattering angle

content. Degradation of the GG backbone starts at ~200°C and results in a 35% weight loss for G1, 20% for G2, and 10% for G3. Degradation of the PEtOx grafts happens within 300 to 400°C. The char yield of the samples follows the GG content with the highest yield found for G1. The derived weight losses of the graft copolymers (Figure S9) show that the fastest losses happen at somewhat lower temperatures than those for the pure polymers. Also, the lower temperature process (GG backbone) splits into two with increasing grafting density. Very similar results were obtained for corresponding physical mixtures of GG and PEtOx, see Figure S10. In the derived thermogram, the splitting of the first process (GG degradation) is present too and therefore it does not originate from grafting. Therefore, more solid proofs of successful grafting are needed.

The grafting of PEtOx to the GG backbone was confirmed using G2 sample by means of diffusion-ordered spectroscopy (DOSY), see Figure S11. The diffusion coefficients (D) were measured for pristine GG, PEtOx chains and for the GG-g-PEtOx copolymers. The calculated diffusion coefficient for GG was 9.2×10^{-12} $m^2\ s^{-1}$ whereas $D = 7.5 \times 10^{-11}$ $m^2\ s^{-1}$ for the corresponding PEtOx, which is close to the value obtained for the G2 copolymer ($D = 7.9 \times 10^{-11}$ $m^2\ s^{-1}$). The larger diffusion coefficient determined for the copolymer in comparison the diffusion of the

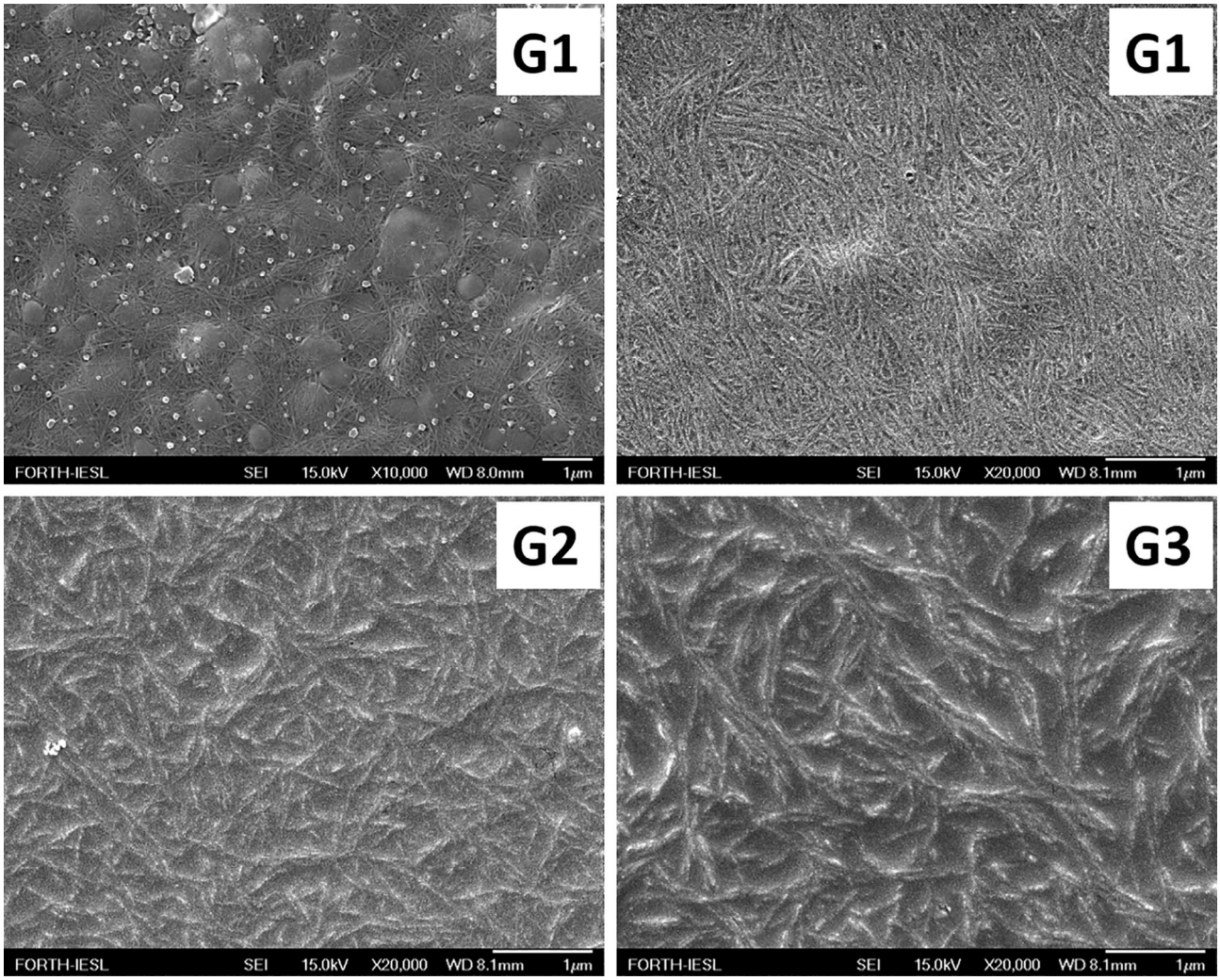

FIGURE 5 SEM images of the dry films composed of graft copolymers. Images for G1 were taken using 10k (left) and 20k (right) multiplication. Images for G2 and G3 are taken with 2k multiplication

unmodified GG could suggest the partial degradation of the GG backbone. Degradation of GG was observed during grafting at 70°C and was confirmed by FT-IR and NMR. A more compact structure of the copolymer chain in comparison to GG can also explain the bigger diffusion coefficient of the copolymer.

Interestingly that dilute aqueous solutions of the G1 copolymer are somewhat opaque or cloudy, which is not observed for solutions of G2 and G3. The optical transmittance of the graft copolymers does not change significantly upon heating in the range of 20–80°C (Figure S12). Opacity may be a sign of particle formation and suggests the existence of specific interactions between GG and PEtOx (e.g., via H-bonding). This is partially confirmed by the DLS results (Figure 4). Pristine GG shows a bimodal size distribution, which is typical for charged, highly swollen and interacting coils. The distributions of the copolymers are broad but monomodal with the peak, which corresponds to the size of the individual GG molecules. Taking into account the stronger scattering from the G1 copolymer solutions, the particles formed by G1 are expected to be denser than the coils of GG and the particles formed by G2 and G3. From the above, it is evident that the degree of grafting affects the solution properties of the synthesized copolymers.

In order to visualize the apparent difference between the GG-g-PEtOx copolymers, SEM images of the G1, G2, and G3 samples were taken. Figure 5 clearly demonstrates that the packing density of dried G1 is significantly higher than that for G2 and G3 and decreases with increasing grafting. Moreover, spherical particles are always present in the images of G1.

The nature of the interaction between GG and PEtOx and possible partial degradation of the GG backbone in the course of the

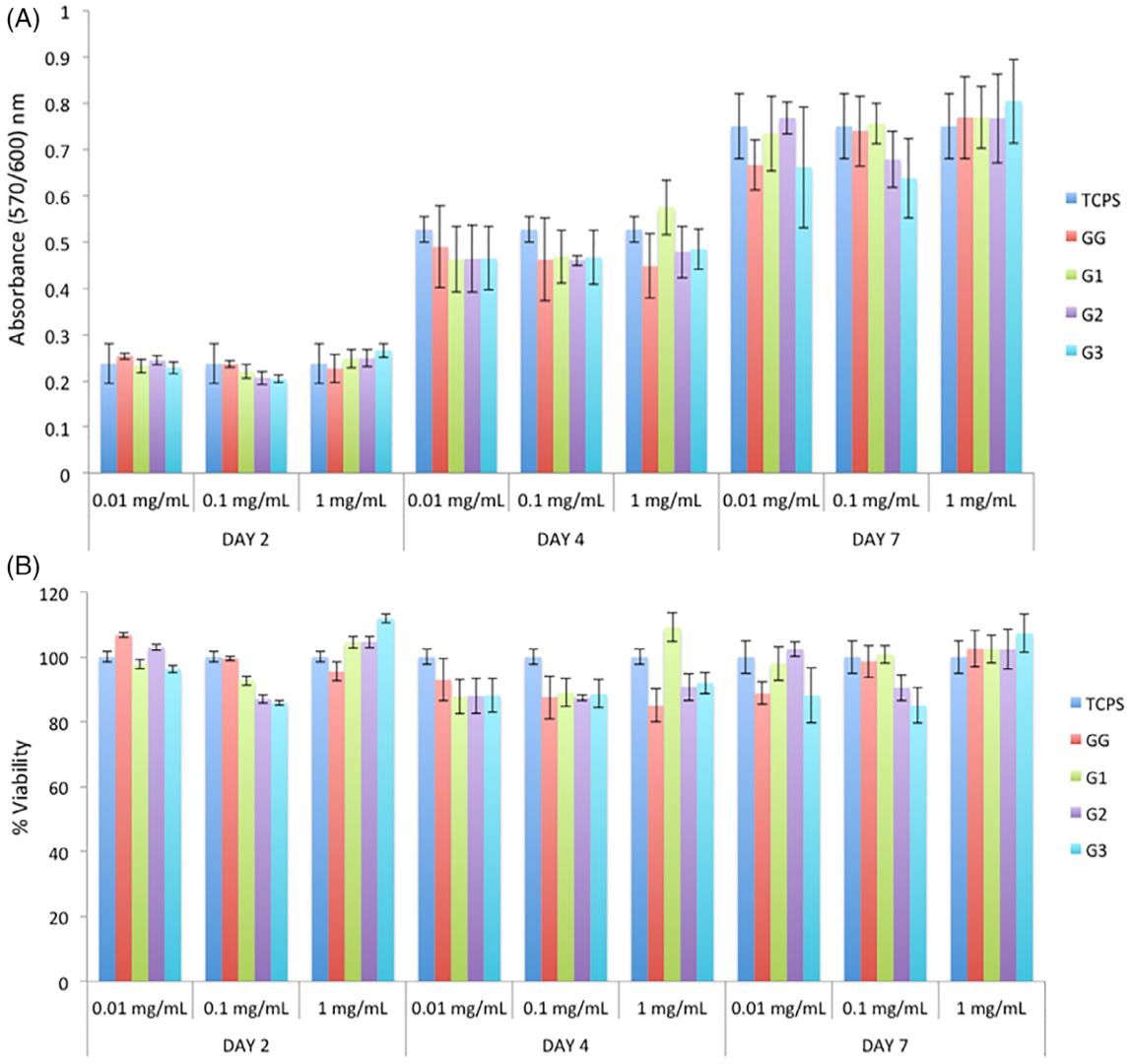

FIGURE 6 Cytotoxicity assessment of gellan gum (GG) and the GG-g-PEtOx copolymers (G1, G2, and G3). Cell viability and proliferation (A) and percentage viability compared to the TCPS control (B) after 2, 4, and 7 days in culture under the induction of 0.01, 0.1, and 1 mg/mL of the polymers

grafting reaction will be investigated further and the synthesis will be optimized and improved.

3.2 | In vitro biocompatibility assessment

In vitro testing was performed in order to assess the potential of the synthesized GG-g-PEtOx copolymers for ocular drug delivery. The validation of the biocompatibility of GG and the GG-g-PEtOx copolymers at concentrations of 1, 0.1, and 0.01 mg/mL was performed by the investigation of the cell morphology, viability, and proliferation using L929 murine fibroblasts. The results indicate that cells adhere strongly from the first day in culture under the induction of all polymers and concentrations, depicting their characteristic elongated fibroblast morphology. The images of Figure S13 indicate that cell morphology in the presence of the materials at the concentration of 1 mg/mL is similar to this on the TCPS control, without any differences among the various materials. On day 2, as the initial cell number is low, single cells adhered on the surface are visible, whereas on day 7, the number of proliferated cells is so high that they form a confluent layer of densely populated cells.

The cell viability was quantified at the polymer concentrations of 0.01, 0.1, and 1 mg/mL using L929 murine fibroblasts after 2, 4, and 7 days in culture in direct contact with the polymers, and the % viability to the TCPS control (set at 100%) are graphically presented in Figure 6. We observe a significant twofold increase in cell proliferation from day 2 to day 4 ($p = 0.0007$), and a similar increase from day 4 to day 7 ($p = 0.002$) in culture independent of the applied concentration as depicted in Figure 6A. Additionally, cells proliferate from day 2 to day 7 in a similar manner under the induction of the materials as on the TCPS control. All materials present a cell viability of at least 85% compared to the TCPS control at all three time points, at all three investigated concentrations as shown in Figure 6B. According to the ISO 10993-5:2009 standard, a limit of 70% cell viability considers the polymers cytocompatible. As all the investigated polymers demonstrate viability above this value, they are highly biocompatible. Statistical analysis indicates no significant differences among the materials concentrations at each time point ($p > 0.1$), and no significant differences between unmodified and grafted gellan gum ($p > 0.5$).

Similar to our study, cell metabolic activity assessment on hydrophilic nanogels based on partially hydrolyzed poly(2-ethyl-2-oxazoline) evidenced that they are not cytotoxic when investigated in a concentration range from 0.1 to 400 μg/mL.[39]

4 | CONCLUSIONS

In this research, we have synthesized biologically safe polymeric carriers for topical drug delivery based on graft copolymers of GG, an anionic polysaccharide that forms transparent gels in situ in water and in the presence of metal ions, and can bind drug molecules, and PEtOx chains, which enable the penetration of the copolymers in the human tissues.

We report the successful synthesis of GG-g-PEtOx copolymers of three different degrees of grafting. A feasible purification procedure for commercial low acyl GG was developed. GG was purified prior to modification, which removed gelling impurities and significantly improved its solubility in DMSO. The synthesis procedure reported for grafting of other polysaccharides was significantly altered and applied to GG. The copolymers were investigated by means of NMR, FT-IR, thermogravimetric analysis, and SEC. The characteristic peaks of GG and PEtOx were detected in the ^{1}H NMR and FT-IR spectra of the GG-g-PEtOx copolymers and the peaks intensities confirm the different grafts content. DOSY NMR proved that the PEtOx grafts are actually attached to the GG backbone. Unexpected intramolecular interactions between the GG backbone and the PEtOx grafts were observed which should be considered in the further development of the drug vehicle. In this respect, the more compact conformation of GG-g-PEtOx in comparison to unmodified GG might be very practical. However, a possible degradation of the backbone and these specific interactions between GG and PEtOx require further investigation and the optimization of the synthetic approach. Further characterization of GG and the graft copolymers by asymmetric field flow fractionation may also be useful.

The ability of GG to form transparent gels in situ triggered by metal ions, which are naturally present in tear fluid can be exploited in the application of the copolymers to prolong the drug retention time on the surface of the eye. Our in vitro tests validate that the investigated GG-g-PEtOx copolymers are highly cytocompatible as they demonstrate a physiological cell morphology as well as an increase in the viability and proliferation of cells cultured under their induction. These results confirm that the proposed materials may further be developed for use in ocular drug delivery. At this stage, our concept is based on gellan as a natural drug delivery vehicle.[40] We expect that PEtOx grafts enable penetration of GG-g-PEtOx into the eye mucus whereas GG binds and carries the drug. Future studies will evaluate the effect of PEtOX grafts on the mucoadhesive properties of gellan gum. It is expected that these properties will be inhibited similarly to previously reported effect of PEtOX on mucoadhesion of thiolated silica nanoparticles[41] and this should lead to enhancement in mucus-penetrating properties. Ultimately, the performance of both types of materials will be compared for the ability to delivery drugs to the eye.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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