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# Biodegradation *in vitro* and retention in the rabbit eye of crosslinked poly(1-vinyl-2-pyrrolidinone) hydrogel as a vitreous substitute

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**Abstract:** To elucidate the relatively short retention of crosslinked poly(1-vinyl-2-pyrrolidinone) hydrogels in the eye when used as potential vitreous substitutes, a  $^{14}\text{C}$ -labeled hydrogel was produced and subjected to both *in vitro* biodegradation assays and *in vivo* experiments. The polymer was synthesized by the free-radical copolymerization of 99% 1-vinyl-2-pyrrolidinone with 1%  $^{14}\text{C}$ -methyl methacrylate in the presence of ethylene glycol dimethacrylate (0.1%) as crosslinking agent. The *in vitro* protocol for assessing the biodegradation included the incubation of hydrogel with hydrolases (trypsin or collagenase), followed by examination of changes in its physical characteristics and by monitoring its residual radioactivity, as well as by detection of possible degradation products. Within the maximum duration of experiments (4 weeks), none of the procedures in-

dicated biodegradation of polymer. The hydrogel was also injected into the vitreous humor of rabbits and followed up to 4 weeks. Residual radioactivity measurements of the vitreous contents indicated that 50% of the polymer was removed by the end of this period. Histopathologic examination revealed cell infiltrates of the mononuclear phagocyte system in both vitreous and retinal tissue. A possible phagocyte-mediated mechanism for the dissipation of hydrogel is discussed. © 1998 John Wiley & Sons, Inc. *J Biomed Mater Res*, **39**, 650–659, 1998.

**Key words:** artificial vitreous;  $^{14}\text{C}$ -labeled poly(1-vinyl-2-pyrrolidinone); biodegradation; hydrogel retention; phagocytosis

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## INTRODUCTION

Research on developing a material as a long-term vitreous substitute in the eye has been carried on for nearly 100 years by scientists and ophthalmologists. Unfortunately, there is no material available yet to function as a permanent replacement for the vitreous body. Such a material would significantly reduce the incidence of blindness caused by eye injuries and vitreoretinal diseases.<sup>1–3</sup> Apart from various postoperative complications inherent to all implants in the eye, one of the major additional problems with the current vitreous substitutes is their short-lived residence in the vitreous cavity.

Recent reviews<sup>4–6</sup> on the history of the artificial vitreous body show that the traditional protocol used to

assess the suitability of various materials as potential vitreous substitutes consisted of selecting a transparent and injectable material and then subjecting it to biocompatibility tests *in vivo*. No *in vitro* (neither cytotoxicity nor biodegradability) evaluation was attempted prior to experiments in animals or trials in human patients. Following an extensive study<sup>7</sup> of poly(methyl acrylamidoglycolate methyl ether) hydrogel as a potential vitreous substitute, which eventually was proved to elicit severe cytotoxic reaction, we were the first to propose and test *in vitro* the cytotoxicity of materials as vitreous substitutes prior to any animal experiments.<sup>8</sup>

We now suggest that the *in vitro* evaluation of material biodegradability before *in vivo* experiments will also reduce animal sacrifices. A readily biodegradable polymer would be of little use as a truly functional replacement for the vitreous because of its short retention in the eye and owing to the release of possible toxic biodegradation products. At the best, such materials can be employed as temporary and partial replacements, but more convenient agents are currently in use for this purpose, such as gases, physiologic sa-

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line solutions, and silicone oils. The first two substances dissipate naturally after a short time; the silicone oils have to be removed from the vitreous after a definite time to avoid serious complications.

The natural clearance pathways of the water from the vitreous should play an essential role in the dissipation of foreign substances. A very active water movement was demonstrated in the vitreous of rabbit,<sup>9</sup> to the extent that half of the water was exchanged in about 15 min, which corresponds to a flow of 85 mm<sup>3</sup>/min. The discrepancy between experimental data using titrated water and a theoretical model based on passive diffusion was interpreted<sup>10</sup> as proof of the existence of an independent flow in the vitreous. Investigations based on the use of various tracer substances<sup>11-14</sup> indicated that (a) the fluids are cleared from the animal or human vitreous mainly through the retinal and choroid vasculature (in excess of 90%) and the remaining by way of anterior chamber and sclera, and (b) the outward clearance from the vitreous greatly exceeds the inward movement of fluids from the choroid. Such a transport mechanism has probably a purposeful role in maintaining normal retinal attachment.<sup>11,14</sup> Clearly, the posterior drainage through retina, choroid, and sclera vasculatures is the major route of clearance of fluids from the vitreous. More particular clearance pathways were confirmed in the rabbit vitreous by Hayreh<sup>15,16</sup> using colloidal iron as a tracer. The posterior drainage took place from the vitreous along the retinal artery within the optic nerve, with extension into the loose perivascular tissue, and then out into the orbit, while only a fraction of the fluid was cleared through the anterior chamber. However, the posterior pathway via the optic nerve could not be detected in the eyes of primates (monkeys or humans).<sup>16</sup> Any polymeric vitreous substitute delivered to the host vitreous body either as an aqueous solution or as a suspension of particulate substance will readily dissipate through any of the described routes regardless of its biodegradability.

There are very few qualitative or quantitative studies on the fate of foreign materials injected into the vitreous humor. The disappearance of various gases injected into the vitreous cavity was usually estimated by ophthalmoscopy<sup>17-23</sup> or by direct measurement of the volume after the entrapment in a device of the injected gas.<sup>24</sup> The retention of some gases<sup>25</sup> and of polyacrylamide<sup>26</sup> was estimated by labeling the materials with radioisotopes, followed by measurements in a scintillation counter. Chemical analytical methods were used to determine the disappearance from the vitreous cavity of rabbits of polygeline (a modified gelatine)<sup>27</sup> and hydroxypropyl methylcellulose.<sup>28,29</sup> The fate of collagen, hyaluronan, and their mixtures in the vitreous humor was investigated by labeling the biopolymers with fluorescein isothiocyanate and then using a fluorophotometric method.<sup>30</sup> Infrared spec-

troscopy was used to investigate the behavior of a radiation-crosslinked poly(vinyl alcohol) gel in the rabbit vitreous,<sup>31-34</sup> with inconclusive results.

In our quest for a permanent vitreous substitute, we have developed synthetic insoluble hydrogels through the free-radical polymerization and crosslinking of 1-vinyl-2-pyrrolidinone (VP).<sup>5,6,35-39</sup> A number of these hydrogels behaved as viscoelastic materials with very high equilibrium water contents; they were optically clear and displayed sufficient resilience after passing through injection needles.<sup>35-39</sup> They also showed no cytotoxic effect *in vitro*<sup>8,38</sup>; on the contrary, some poly(1-vinyl-pyrrolidinone) (PVP) gels showed protective and growth-promoting effects on fibroblasts in static cultures.<sup>40</sup> However, when the retention in the rabbit eye of a selected hydrogel was investigated by Fourier transform infrared (FTIR) spectrometry, it was found<sup>35</sup> that about 80% of the injected material disappeared from the vitreous cavity within 1 month, which is rather unexpected from a crosslinked, insoluble polymer. Preliminary testing of biodegradation was not contemplated in our previous studies, since PVP is known to be biostable in living organisms when administered as an aqueous solution<sup>41</sup>; high molecular PVP is distributed throughout the body and eventually stored in various organs, while the lower molecular macromolecules are passively excreted. There is no study available on the biodegradation of crosslinked PVP. The present experiments were designed to assess this possibility and to shed more light on the short residence life of PVP gels in the vitreous body.

## MATERIALS AND METHODS

### Materials

<sup>14</sup>C-Labeled sodium cyanide (<sup>14</sup>C-NaCN) was obtained from DuPont Australia Ltd. VP was provided by BASF Australia Ltd. as a monomer, with a purity of 99 wt %. Prior to polymerization, VP was additionally purified by vacuum distillation (b.p. 48°C/2 mm Hg). Ethylene glycol dimethacrylate (EGDMA) from Tokyo Kasei Kogyo Co. (Japan) was used as a crosslinking agent. 2,2-Azo-bis-(2,4-dimethyl valeronitrile), supplied as V-65® by Wako Pure Chemical Industries (Japan), was used as an initiator for the polymerization process.

Trypsin solution (pH 7) containing EDTA (1:250) was purchased from Biosciences Pty. Ltd., Australia, and collagenase (EC 3.4.24.3, Type 1A) from Sigma Chemical Co. (St. Louis, MO), with a specific activity of 305 U/mg. As buffers for dialysis, phosphate-buffered solution (PBS) was used for trypsin, and 0.05 M Tris-HCl solution containing 0.01 M CaCl<sub>2</sub> (pH 7) for collagenase. Slide-A-Lyzer™ dialysis cassettes (retention molecular weight 10 kDa; volume capacity 0.5-3 mL) were supplied by Pierce Chemical Co. (Rockford, IL). TLC (20 × 20 cm) F254 plates were purchased from

Sigma-Aldrich Ltd. (Australia). LKB scintillation cocktail (OptiPhase HiSafe 3) was purchased from LKB Instruments Ltd. (Finland).

### Synthesis of $^{14}\text{C}$ -methyl methacrylate ( $^{14}\text{C}$ -MMA)

The preparation procedure of  $^{14}\text{C}$ -MMA from  $^{14}\text{C}$ -NaCN was a modified version of the method described in several papers<sup>42-44</sup> (Scheme I).  $^{14}\text{C}$ -Aceton cyanohydrin was collected as a fraction with b.p.  $60^\circ\text{--}62^\circ\text{C}/6$  mm Hg (yield 74.7%).  $^1\text{H-NMR}$ ,  $\delta$  (ppm): 1.6 (s, 6H,  $\text{CH}_3$ ); 4.2 (s, 1H, OH).  $^{14}\text{C}$ -MMA was obtained as a clear liquid (b.p.  $96^\circ\text{--}98^\circ\text{C}$ ; yield 46%).  $^1\text{H-NMR}$ ,  $\delta$  (ppm): 2.0 (s, 3H,  $\text{CH}_3\text{-C}=\text{C}$ ); 3.7 (s, 3H,  $\text{CH}_3\text{-O-COR}$ ); 5.6 (s, 1H,  $\text{H}_{\text{trans}}\text{-C}=\text{C}$ ); 6.1 (s, 1H,  $\text{H}_{\text{cis}}\text{-C}=\text{C}$ ).

One milliliter of labeled monomer was mixed into a scintillation vial with 3 mL OptiPhase Hisafe scintillation liquid. Its activity was tested using an LKB Wallac 1209 liquid scintillation counter. Three specimens from the same sample were measured, each in triplicate. The radioactivity was  $1.8 \times 10^6$  dpm/mg.

### Preparation and physical characterization of $^{14}\text{C}$ -labeled hydrogel

The procedures for preparation and physical characterization of  $^{14}\text{C}$ -labeled PVP hydrogel ( $^{14}\text{C}$ -EM1) are similar to those described elsewhere.<sup>35,37-39</sup> The weight composition of the polymerization mixture was 99% VP and 1%  $^{14}\text{C}$ -MMA. EGDMA and V-65 were added as crosslinking agent and initiator (0.1 wt % of total monomers, respectively).

Labeled hydrogel (0.5 mL) was placed in a scintillation vial, dried at  $75^\circ\text{C}$  for 12 h, and tested in a scintillation counter. Three specimens from the same sample were measured, each in triplicate. The radioactivity was  $4.5 \times 10^4$  dpm/mL.

### Dynamic mechanical analysis

The mechanical spectra of  $^{14}\text{C}$ -EM1 hydrogel were recorded in a controlled stress rheometer (Bohlin CS-10; Swe-

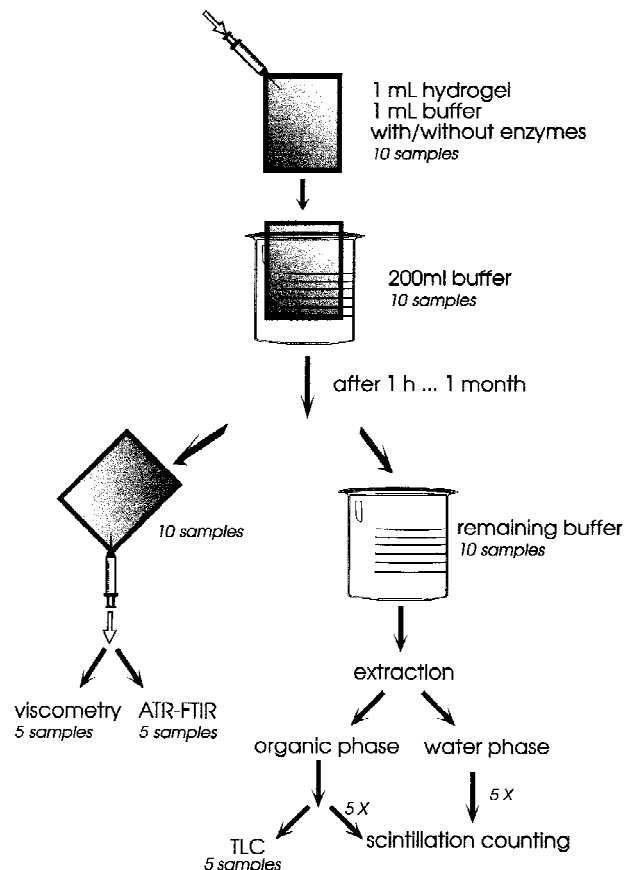
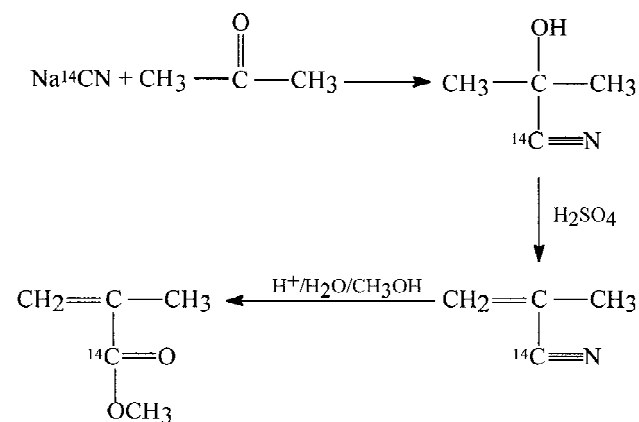
den) at room temperature, as detailed elsewhere.<sup>36,38</sup> Prior to analysis, the yield stress of samples was determined using a ramp stress test, to ensure that the measurement was in the linear viscoelastic region. The gel was placed between a 60-mm-diameter lower plate and a  $4^\circ/40$ -mm-diameter cone truncated to give a gap of 0.15 mm. The frequency of oscillatory shear stress varied from 0.001 to 0.1 Hz, and the strain was kept well below the value of yield strain as provided by the yield stress analysis. The plots of the complex shear modulus (real part as the storage modulus  $G'$  and imaginary part as the loss modulus  $G''$ ) were recorded after the injection of gel.

### Evaluation of biodegradation *in vitro*

The procedure of assessing the biodegradation *in vitro* is illustrated in Figure 1. Ten identical gel specimens were used to allow five samples for the final analyses.

#### *In vitro* treatment

$^{14}\text{C}$ -EM1 hydrogel (1 mL) was freshly mixed with 1 mL enzyme solution (trypsin as supplied or collagenase as a



**Figure 1.** Protocol for *in vitro* assessment of hydrogel biodegradation.

solution 1 mg/mL in Tris-HCl buffer) and injected through a small-gauge needle into a dialysis cassette, which was then placed into 200 mL of appropriate buffer solution (PBS for trypsin and Tris-HCl with 0.1% CaCl<sub>2</sub> for collagenase). The unit was incubated at 37°C in 5% CO<sub>2</sub> atmosphere for five different time lengths: i.e., 1 h, 1 day, 1 week, 2 weeks, and 4 weeks. Fresh enzyme (1 mL) solution was injected into the dialysis cassette every 4 days. For each time point, control determinations (polymer without enzyme) were also performed. At desired time points, the contents of the cassettes and the beakers were subjected to further processing. Attenuated reflectance (ATR) FTIR spectroscopy was used to analyze the contents removed by syringe aspiration from cassettes. The buffer solution, incorporating possibly leached compounds, was extracted with ether. The organic phase was subjected in each case both to TLC analysis and to scintillation counting. The water phase was subjected only to scintillation counting.

### ATR-FTIR spectrometry

The contents in the dialysis cassette after 1 h (as control) and 4 weeks were collected by aspiration through a syringe with a small-gauge needle, and their spectra were recorded in a Bruker FTS-66 spectrometer following a previously described technique.<sup>35</sup> The spectra of the buffers (PBS and Tris-HCl), trypsin solution, and collagenase solution were also recorded. The spectra of gels were corrected by subtraction of spectra of the appropriate buffer and/or enzyme.

### Viscosity measurements

After treatment for 4 weeks, the gels were subjected to stress viscometry measurements using stepped shear stress. The Bohlin controlled stress rheometer was used for all measurements, performed at room temperature. The samples were taken out from the dialysis cassettes through a small-gauge needle, and delivered to the instrument port by injection. Eighteen individual shear stresses were selected between 0.06 and 3.3 Pa, and the viscosity of the specimen was then recorded according to each shear. The viscosity of the control samples (gels without enzymes) was also determined.

### Thin layer chromatography

After removing the dialysis cassette, the remaining buffer solution was extracted five times with 50 mL ether. The combined organic phase was then dried over anhydrous magnesium sulphate and concentrated to 1 mL.

Five of the above extracts were analyzed by TLC. Each sample was spotted in the same volume on TLC plates. The plates were then developed in four different developing solvents, including chloroform, hexane, chloroform/

methanol (3:1), and chloroform/methanol/hexane (4:1:2). Short- and long-wavelength ultraviolet light and iodine vapor staining were used to detect the possible components.

### Radioactivity measurements

The remaining five concentrated organic extracts were subjected to measurements of residual radioactivity. One milliliter of organic phase was mixed with 3 mL scintillation liquid and placed in the scintillation counter.

The water-phase portions (about 200 mL each) were concentrated to 15 mL. Aliquots of 3 mL each and 3 mL scintillation liquid were mixed into vials and placed in the counter. The radioactivity of scintillation liquid (background) served as a control.

### Evaluation of gel retention in the eye

#### Surgical procedure

The surgery involved in this study was similar to that described in our previous studies<sup>7,35,37,38</sup> and was conducted in strict accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990). Briefly, 0.5 mL hydrogel was injected into the right eyes of six New Zealand half-lop rabbits. The follow-up time points were 1 h, 1 day, 1 week, 2 weeks, and 4 weeks, respectively. After sacrifice and enucleation, the eye was sliced open and the content of the vitreous cavity (a mixture of hydrogel, partly liquefied vitreous, solid matter, and physiologic fluids) was carefully collected into a scintillation vial. The empty cavity was then washed with water several times, and the aqueous portions were collected into the same vial. The contents collected 1 h after insertion were used as controls.

#### Radioactivity measurements

The vials containing the materials removed from the eyes were dried at 75°C for 12 h. Scintillation liquid (3 mL) was added to each vial, and the samples were then subjected to scintillation counting. The results from six animals for each time point were averaged.

#### Histopathology

The methodology for the histopathologic examination of animal eyes containing vitreous substitutes has been described in detail in our previous reports.<sup>7,37,38</sup> The eye cups were fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer. Semithin 2- $\mu$ m sections were cut using an ultramicrotome and stained with Toluidine blue for light microscopy. Ultrathin sections (0.1  $\mu$ m) were cut from selected areas and stained with uranyl acetate and lead citrate for transmission electron microscopy (TEM).

## RESULTS

### Physical properties

Physical properties of  $^{14}\text{C}$ -EM1 hydrogel are summarized in Table I. The hydrated polymer had a high water content and was clear and transparent. It also displayed viscoelastic behavior and a certain cohesiveness. The extraction and sterilization process did not alter its physical appearance and properties. The hydrogel passed easily through a small-gauge needle. After injection, the fragmentation of the gel was minor and its transparency did not change significantly.

The dynamic mechanical spectrum of the polymer after a single injection is shown in Figure 2. The storage modulus ( $G'$ ) representing the recoverable energy and the loss modulus ( $G''$ ) estimating the dissipation of energy were measured to investigate the viscoelastic behavior of the material. The plots of  $G'$  and  $G''$  were almost parallel, and there was no crossover in the range of frequencies used. This indicated that the gel was still covalently crosslinked after injection, as the storage modulus remained greater than the loss modulus. The material behaved as an elastic gel rather than a viscous fluid.

### ATR-FTIR spectrometry

The partial spectrum of the fully hydrated  $^{14}\text{C}$ -EM1 is shown in Figure 3. As the gel contained around 99% water, the hydrogen-bonding interaction between the polymer and water induced a broad  $\nu(\text{O-H})$  band between  $3000$  and  $3500\text{ cm}^{-1}$  which could not provide accurate information for analysis, and the region above  $1800\text{ cm}^{-1}$  was omitted from our analysis. The region below  $1100\text{ cm}^{-1}$  was unremarkable. As the contents of crosslinking agent (EGDMA) and comonomer (MMA) are quite low, their characteristic bands are of such a low intensity that the spectrum of copolymer  $^{14}\text{C}$ -EM1 is virtually identical to that of PVP. The spectral region was dominated by a strong intensity band at  $1640\text{ cm}^{-1}$  owing to the carbonyl Amide I band with the  $\text{C}=\text{O}$  stretching mode as its major component.<sup>45</sup>

TABLE I  
Physical Properties of  $^{14}\text{C}$ -EM1 Hydrogel\*

Water content	98.4%
Refractive index	1.3445
Transmittance at 550 nm	89.9%
Light transmission	90%
Visual acuity (USAF resolution test target)	2.3

\*Methods are described in Ref. 38.

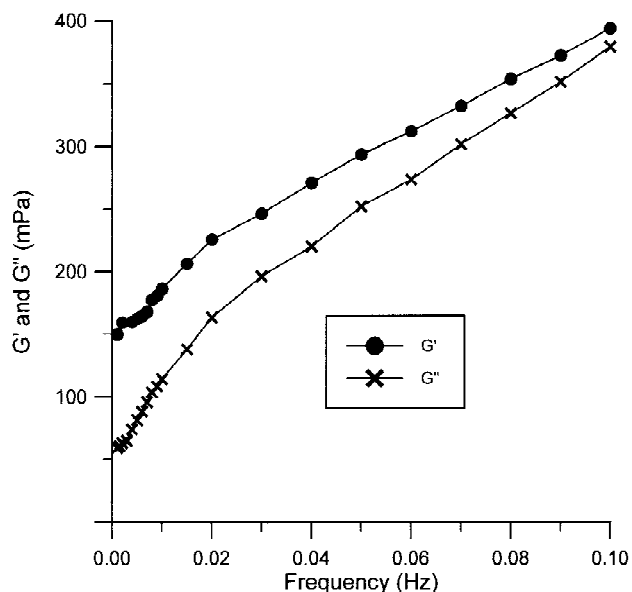


Figure 2. Mechanical spectrum of  $^{14}\text{C}$ -EM1 gel after injection through a 30-gauge needle.

The spectra of the contents removed after 4 weeks from dialysis cassettes are shown in Figure 4 and compared to the spectrum of a control in PBS. There were no significant changes in the intensities of signals of all major bands in any of the spectral regions.

### Viscometric analysis

The viscometric spectra of the specimens after being subjected for 4 weeks to different treatments are compared in Figure 5. Over the range of shear stress investigated, there were no significant differences between the viscosities of the treated samples and the control sample.

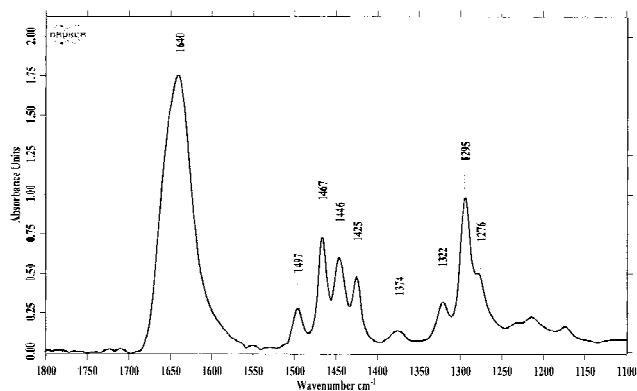
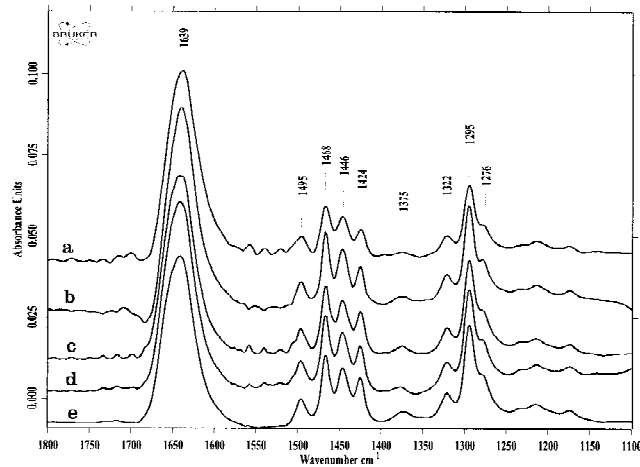


Figure 3. Infrared spectrum of the copolymer VP/ $^{14}\text{C}$ -MMA (99:1) crosslinked with 0.1 wt % ethylene glycol dimethacrylate ( $^{14}\text{C}$ -EM1 gel).



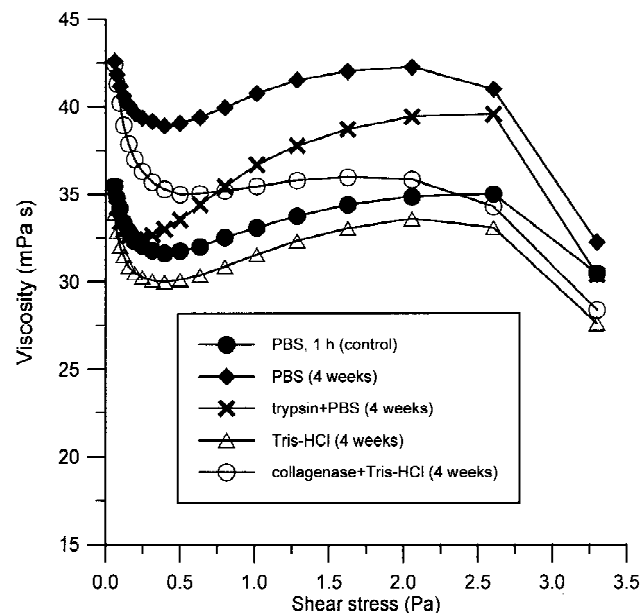
**Figure 4.** Infrared spectra of <sup>14</sup>C-EM1 gel specimens subjected to various treatments: (a) 1 h in PBS (control); (b) 4 weeks in PBS; (c) 4 weeks in Tris-HCl buffer; (d) 4 weeks in presence of trypsin; and (e) 4 weeks in presence of collagenase.

**TLC analysis**

Regardless of the developing solvent or visualization method used, no individual compounds could be detected on the TLC plates in the samples of ether concentrates resulting from the extraction of buffer solutions.

**Radioactivity measurements after dialysis**

The residual radioactivities of the organic extracts and water phase portions resulting from dialysis of



**Figure 5.** Viscosity of <sup>14</sup>C-EM1 gel plotted against frequency of an oscillatory shear stress. Gel specimens were subjected to various treatments as indicated.

gels are shown in Table II. The radioactivity of all samples was very similar to that of the control. The results indicate that there were no resulting <sup>14</sup>C-labeled molecules with a molecular weight <10 kDa available to leach out through the dialysis membrane.

**Radioactivity measurements after *in vivo* experiments**

The radioactivities of the contents of the vitreous cavity removed at certain time points during the experiments in the rabbit eyes are shown in Table III. These values represent a reasonable estimation of the rate of disappearance of the <sup>14</sup>C-EM1 gel from the eye, and they show that within 4 weeks about 50% of the injected gel disappeared from the vitreous cavity.

**Histopathology**

The eyes injected with the <sup>14</sup>C-EM1 hydrogel generally showed the same features as those found previously in the eyes containing other polymers of VP.<sup>37,38</sup> Light microscopic examination of the sections revealed the presence of macrophages after 4 weeks in the vitreous cavity, neural retina, and subretinal space (Fig. 6). TEM showed that the macrophages contained

**TABLE II**  
Specific Residual Radioactivity of Contents Removed from Dialysis Cassettes

Treatment	Time	Radioactivity (dpm)	
		Organic Phase	Water Phase
Control*		25.5 ± 4.3	
PBS	1 h	25.4 ± 2.7	19.6 ± 0.3
	1 day	22.7 ± 1.8	34.8 ± 10.9
	1 wk	27.3 ± 2.9	27.1 ± 2.0
	2 wk	23.9 ± 1.4	21.1 ± 0.3
Trypsin + PBS	4 wk	26.2 ± 3.8	24.6 ± 2.5
	1 h	24.7 ± 2.3	21.6 ± 1.0
	1 day	32.0 ± 9.6	20.8 ± 0.8
	1 wk	22.2 ± 4.6	24.7 ± 3.6
Tris-HCl	2 wk	22.7 ± 2.5	24.7 ± 0.9
	4 wk	26.1 ± 1.6	20.6 ± 2.3
	1 h	22.6 ± 3.3	17.6 ± 3.5
	1 day	46.4 ± 25.4	25.6 ± 2.1
Collagenase + Tris-HCl	1 wk	22.5 ± 1.9	26.1 ± 1.3
	2 wk	29.9 ± 5.1	19.7 ± 0.8
	4 wk	28.5 ± 1.0	27.3 ± 2.3
	1 h	25.2 ± 2.9	18.6 ± 0.7
Collagenase + Tris-HCl	1 day	25.2 ± 0.8	24.5 ± 1.2
	1 wk	28.9 ± 1.1	29.6 ± 2.0
	2 wk	43.5 ± 16.2	25.6 ± 0.4
	4 wk	28.9 ± 1.8	27.1 ± 1.5

\*Scintillation liquid only.

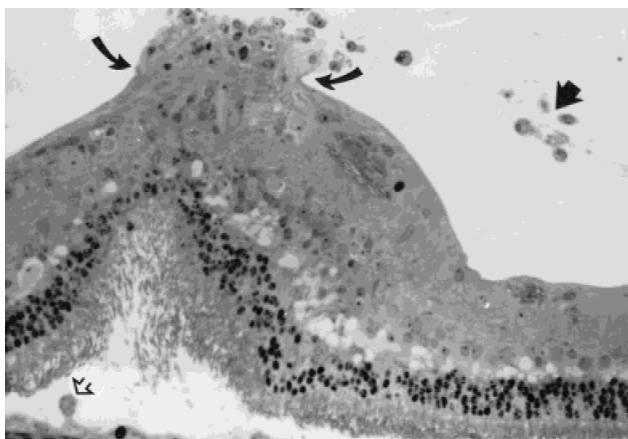
**TABLE III**  
**Specific Residual Radioactivity of Materials Removed**  
**from Vitreous Cavity of the Rabbit Eye**

Follow-up	Radioactivity (dpm)	Estimated Residual Polymer (%)
1 h	10,180 ± 3202	100 (control)
1 day	10,129 ± 2684	99.5
1 wk	7167 ± 2261	70.4
2 wk	6589 ± 1259	64.7
4 wk	4942 ± 1658	48.5

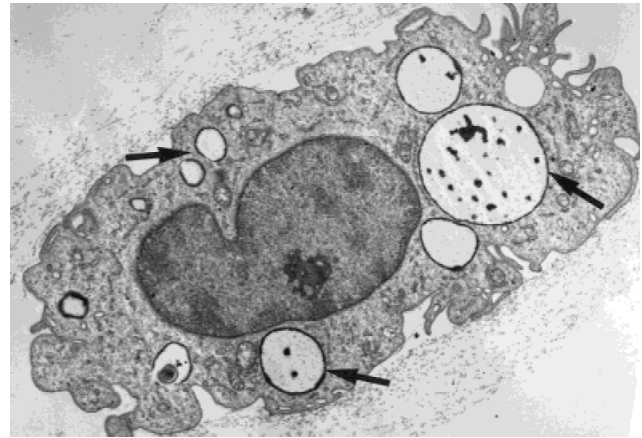
large vacuoles which were very likely polymer particles incorporated in phagolysosomes (Fig. 7). We cannot explain at this stage the nature of the black spots seen in some vacuoles.

## DISCUSSION

The  $^{14}\text{C}$ -labeled crosslinked copolymer VP/MMA displayed desirable physical and mechanical properties as a vitreous substitute. The gel characteristics did not change following the *in vitro* incubation of polymer in two proteolytic enzymes, trypsin and collagenase. Further analyses of the polymer (ATR-FTIR spectrometry) and of the incubation media (TLC and scintillation counting) at various intervals of time during treatment indicated that no biodegradation occurred after 4 weeks. However, the *in vivo* study indicated that 50% of the initially injected hydrogel disappeared from the rabbit vitreous over the same period of time. Previous investigations<sup>35</sup> by ATR-FTIR on a crosslinked homopolymer of VP indicated an 80% loss within the same duration, but this result may be an



**Figure 6.** Light micrograph of the retina showing vacuolated macrophages (arrow) in the vitreous cavity, and a rupture in the inner limiting membrane (between bent arrows). A movement of macrophages between the retina and vitreous is evident. A macrophage (open arrow) is seen in the subretinal space, contiguous with the retinal pigment epithelium. (Original magnification  $\times 300$ .)



**Figure 7.** Transmission electron micrograph of a macrophage in the vitreous cavity. An indented nucleus is seen surrounded by vacuoles (arrow), probably containing synthetic polymer. (Original magnification  $\times 10,000$ .)

overestimation entailed by a probably inappropriate collection of the vitreous contents, and due to the insufficient sensitivity of infrared spectrometry in analyzing such complex mixtures. Nevertheless, the present study corroborates these findings and shows that crosslinked PVP hydrogels display a relatively short-lived residence in the rabbit vitreous.

In previous studies,<sup>35,37,38</sup> we speculated that biodegradation may play a role in the disappearance of PVP hydrogels from the rabbit vitreous despite the well-known biostability of uncrosslinked PVP.<sup>41</sup> In fact, crosslinked PVP should be even more stable. Although the mammalian vitreous body is a medium rich in enzymes,<sup>46-52</sup> we believe now, on the basis of present results, that the enzyme-induced biodegradation of PVP hydrogels does not play any significant role in the process of their disappearance from the vitreous humor, at least not within the relatively short period of time which proved to be sufficient for a massive depletion *in vivo*. It remains that the removal of synthetic material from the vitreous is due to other causes.

Phagocytosis of hydrogels in the rabbits' vitreous was a prominent finding in this study, as in our previous reports.<sup>37,38</sup> The early work of Evans<sup>53</sup> and Berliner and Nonidez<sup>54</sup> has almost been forgotten with the passage of time, but they demonstrated accurately that after the injection of ink into the rabbit vitreous, the ink particles were ingested by phagocytes which transported them out of the cavity through the posterior clearance pathway via the optic nerve. Such a scenario seems reasonable in the present case, too, with the observation that this pathway is not necessarily the major one. The phagocytic transportation appears plausible as a general mechanism, as the migrating phagocytes carrying the ingested polymer can also follow other clearance pathways.

Over the duration of our *in vivo* experiment, it appeared that the digestion of the vacuolized polymer did not actively progress to completion owing to the possible resistance of the polymer to lysosomal enzymes. The use of a synthetic material as a vitreous substitute is probably not precluded in such conditions, provided its transparency and ability to ensure a proper tamponade of the retina against the choroid and to counteract vitreoretinal tractions are all maintained. While the transparency and clarity of the vitreous was not affected following the injection of gel, the fulfillment of the latter requirement seems improbable, as no mechanical strength is to be expected from a gel fragmented to the extent that could be ingested by phagocytes. This brings us to the most intriguing aspect of our present and previous findings—namely, what process could possibly cause the fragmentation of gel into particles sufficiently small (around 2  $\mu\text{m}$ <sup>55</sup>) to be engulfed by phagocytes? In our previous reports,<sup>35,37,38</sup> the biodegradation of gels and/or their shearing during injection were suggested as possible causes. The present results preclude the former alternative. As for the latter, it is known that the process of injection through small-gauge needles subjects a gel to enormous shear stress which can cause massive fragmentation. One of the consequences of this fragmentation would be poor optical characteristics. However, many crosslinked polymers of VP maintain good optical properties after one injection cycle,<sup>39</sup> and this was a major reason for proposing and investigating them as vitreous substitutes. After injection, the fragments of gel mix together rapidly in such a manner that no individual particles are distinguishable. After the insertion of gel into the natural vitreous humor, this organization is likely to be disturbed. In other words, the particles, which are already kept together only loosely because of the enormous volume of water (98–99%) in the system, are introduced suddenly in a dynamic aqueous medium consisting mainly of water (99%) in which a large variety of biopolymers, organic compounds, and ionic compounds are present.<sup>56–59</sup> In these new conditions, the synthetic hydrogel may undergo syneresis (i.e., contraction of gel and concomitant separation of hydration water), and the weak interactions between particles are broken; as a result, the particles become free. They probably float through the vitreous humor until turning into easy prey for the cells of the mononuclear phagocyte system. Our visual observations during processing the enucleated rabbit eyes support this hypothesis. Upon cutting open the vitreous cavity, mostly water could be seen inside, an indication that both natural gel and synthetic hydrogel collapsed through syneresis. The separated solid matter could not be seen macroscopically, as its amount would not exceed 1% of the total content.

Although further research is needed to demonstrate that fragmentation of hydrogel by shearing through a

needle and subsequent syneresis in the vitreous humor can lead to a “phagocytizable” size of the particles, the above hypothetical scenario appears more plausible than previous attempts to explain the short residence life of synthetic hydrogels in the vitreous body. However, it is doubtful as to how far these conclusions are applicable to humans, as the clearance pathways, and therefore the rate of dissipation, of fluids in the human vitreous are believed to be different in some respects from the rabbit vitreous.

## CONCLUSIONS

Although a crosslinked copolymer VP/<sup>14</sup>C-MMA was not biodegraded *in vitro* by two proteolytic enzymes (hydrolases) over 4 weeks, half of it disappeared from the rabbit vitreous within the same period of time. The histopathologic findings suggest a phagocyte-mediated mechanism for the removal of polymer from the vitreous cavity.

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