Doklady Biochemistry and Biophysics, Vol. 405, 2005, pp. 445–449. Translated from Doklady Akademii Nauk, Vol. 405, No. 5, 2005, pp. 686–691.

BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Heterogeneity of Structure and Fluorescence of Single Lipofuscin Granule from Retinal Pigment Epithelium of Human Donor Eyes: Study with the Use of Atomic Force Microscopy and Near-Field Microscopy

A. N. Petrukhin*, A. A. Astaf'ev*, P. N. Zolotavin*, T. B. Fel'dman, A. E. Dontsov**, O. M. Sarkisov*, and Academician M. A. Ostrovsky****

Received September 16, 2005

INTRODUCTION

The phagosomes which were not digested completely by lysosomal enzymes are accumulated into lipofuscin granules during aging of retinal pigment epithelium (RPE). In turn, the phagosomes consumed by RPE cells contain debris of outer segments of retinal rods and cones. The size of a single lipofuscin granule is 0.5×1.0 micron. In addition to the remains of proteins and lipids, it contains more than ten fluorophores and photosensitizers which, probably, are the products of conversion of all-*trans*-retinal which is released from the molecule of visual pigment due to pigment photolysis.

Chemical nature was established only for two fluorophores. They are bis-retinylidene ethanolamine (A2E) [1] and the dimer of all-*trans*-retinal with phosphatidylethanolamine [2]. Under the exposure to visible light, lipofuscin granules generate reactive oxygen species which, in turn, may damage proteins, lipids, and DNA and initiate apoptosis in of pigment epithelial cells [3–6]. It was found that the development of degenerative deceases of the retina correlates with the accumulation of lipofuscin granules in the RPE cells, and light may intensify progression of these deceases. It is possible to hypothesize that the damaging effect of light on the RPE cells is determined mainly by the photochemical activity of lipofuscin granules and their fluorophores. Therefore, it is important to study the structural, spectral, and photochemical characteristics of lipofuscin granules from the RPE cells to understand the pathogenesis of these severe eye diseases and to develop prophylactic measures against them and, presumably, the methods of their treatment.

In this study, we investigated the ultrastructural organization and fluorescent properties of single lipofuscin granules by the methods of atomic force microscopy and near-field microscopy. We found that the inner structure and the distribution of fluorescence in the granule were not homogeneous. Note that the fluorescence of lipofuscin granule during irradiation with visible light was decreased.

MATERIALS AND METHODS

Preparation of lipofuscin granules. Lipofuscin granules were obtained from retinal pigment epithelium (RPE) of eyes of 50- to 75-year-old donors as described in [3, 4]. Donor eyes were obtained from the eye bank of Moscow Institute of Eye Microsurgery. The donor eyes did not have any ophthalmologic deceases. The RPE cells were separated from the tissue with the aid of a coarse brush, placed in phosphate buffer, and sonicated at 4°C for 60 s at a frequency of 22 kHz and the maximum resonance. The undestroyed remains of RPE cells were removed by centrifuging at $60g$ for 10 min. The supernatant was centrifuged at $6000g$ for 15 min to pellet pigment granules. The pellet of granules was suspended in 0.3 M sucrose, layered on a sucrose density gradient (molar densities, 2.00 : 1.80 : 1.60 : 1.55 : 1.50 : 1.40 : 1.20 : 1.0), and centrifuged at 103000 g for 1 h. The pellets containing lipofuscin granules (1.0–1.4) were collected and centrifuged in the sucrose density gradient. Finally, lipofuscin granules were washed three times from sucrose by 0.1 M phosphate buffer. The concentration of granules was determined in the Goryaev chamber by the standard method. The initial concentration of granules was 3×10^8 granules/ml. In the experiments, lipofuscin granules were resuspended in 0.1 M potassium phosphate buffer (pH 7.3).

* *Semenov Institute of Chemical Physics, Russian Academy of Sciences, ul. Kosygina 4, Moscow, 117977 Russia*

** *Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, ul. Kosygina 4, Moscow, 117977 Russia*

1607-6729/05/1112-0445

To prepare samples for microscopy, the suspension of lipofuscin granules was diluted with water in 100–200 times and placed in the ethanol-containing solution. A drop of the granule suspension was placed on a cover slip which was then placed in a desiccator for drying.

Atomic force microscopy and near-field microscopy. The experimental device was made with the use of an Olympus IX71 inverted optical microscope equipped with the heads of an atomic force microscope or a near-field microscope. A silicon needle (radius, approximately 10 nm) was used as a probe in the atomic force microscope. We used a semi-contact mode which may be used to study soft biological samples.

Single-mode optic fiber whose end was sharpened by chemical pickling was used as a probe for near-field microscope. The fibers were covered with a thin aluminum layer which reflects the most part of light back into the fiber to prevent discharge of light through the fiber sides. An aperture with a diameter about 100 nm was made on the fiber end. The use of such a small aperture allowed us to achieve spatial resolution which was less than the wavelength.

The second harmonic (420 nm) of a femtosecond laser or the irradiation of a continuous-wave laser (wavelength, 532 nm) was applied to the free end of the fiber. The power applied to the fiber was less than 1–3 mW to prevent the destruction of the sharpened part of the probe. The light coming through the sample and probe aperture was collected by the microscope objective (40×, $NA = 0.65$) and recorded in each point of the sample with the use of a photo amplifier (PA). The map of optical transmission of the sample was obtained by successive recording of light coming through each point of the sample. An Acton SP–300 polychromator and a Pi–MAX highly sensitive PZS camera with a brightness amplifier were used as a detector instead of PA to record the fluorescence spectra in the single points of the sample.

RESULTS

Atomic Force Microscopy

Figure 1a shows the image of a single lipofuscin granule. The size of scanning region was 4.1×4.1 μm; the height of the granule, 700 nm; and the diameter of the bottom, 2.9–3.3 nm. The granule had two "humps". The distance between them was 800 nm; the depth of hole between them, 100 nm. Such shape and characteristic sizes were typical of all lipofuscin granules studied. A similar shape of the granules was also described in [7].

Figure 1b shows the image obtained during scanning of the central region of the granule by means of phase contrast. Two types of formations with characteristic sizes of 100–120 nm or 30–40 nm are seen in the image. The method of phase contrast may provide additional data on rigidity of the granule material. The granule contains mainly a soft material (shown with dark colors), whereas it central part contains aggregates of a more rigid material (shown with bright colors).

Near-Field Microscopy

Figure 2a shows the height profile of a single granule obtained with the aid of a near-field microscope. We marked six points in which we recorded the fluorescence spectra. All points were in the central part of the granule. The distance between them varied from 0.5 to 3.5 μm.

As it was mentioned, the sample fluorescence was excited with the use of a probe fiber of the near-field microscope. A very small volume is excited in each point, therefore, it is necessary to measure very small fluorescent signals. A PZS camera equipped with a brightness amplifier and cooled by Peltier elements was used to record these signals. However, the fiber which was used to prepare the probe had self-fluorescence. We recorded self-fluorescence of the fiber during the application of 420-nm light to the fiber. However, the intensity of this parasitic signal was comparable with the intensity of the useful signal (the sample fluorescence) and was shifted a little towards the longwave range. The fluorescence spectra of lipofuscin granule were obtained by subtraction of the baseline signal. We are planning to eliminate self-fluorescence of the probe fiber by selection of a special optical fiber.

As shown on Fig. 2a, the fluorescence spectra at the points *1–3* were very similar by both shape and intensity. The maximum of these spectra was at 575 nm. The spectrum with a similar shape was observed in the point *6*; however, its intensity was smaller almost by an order of magnitude. We did not observe any fluorescence in the point *5*. A small signal with the maximum at 675 nm was recorded at the point *4*. These data suggest that the fluorophore distribution in a single lipofuscin granule is heterogeneous.

Figure 2b shows a decrease in the fluorescence intensity during long-term irradiation of several lipofuscin granules with green light (wavelength, 532 nm). Samples were excited using the probe of the near-field microscope. The fluorescence spectra recorded after a continuous irradiation with increasing light doses from 0 to 24×10^5 J/cm^2 are shown. It is seen that an increase in the dose considerably decreased the fluorescence intensity. Moreover, the fluorescence spectra were changed. These data indicate that irradiation of lipofuscin granule at 532 nm induces a decrease in the concentration of fluorophores, their bleaching and conversion into the products absorbing light shorter than 420 nm. A change in the spectrum shape may be determined by the presence or appearance in the granule of fluorophores which differ from A2E.

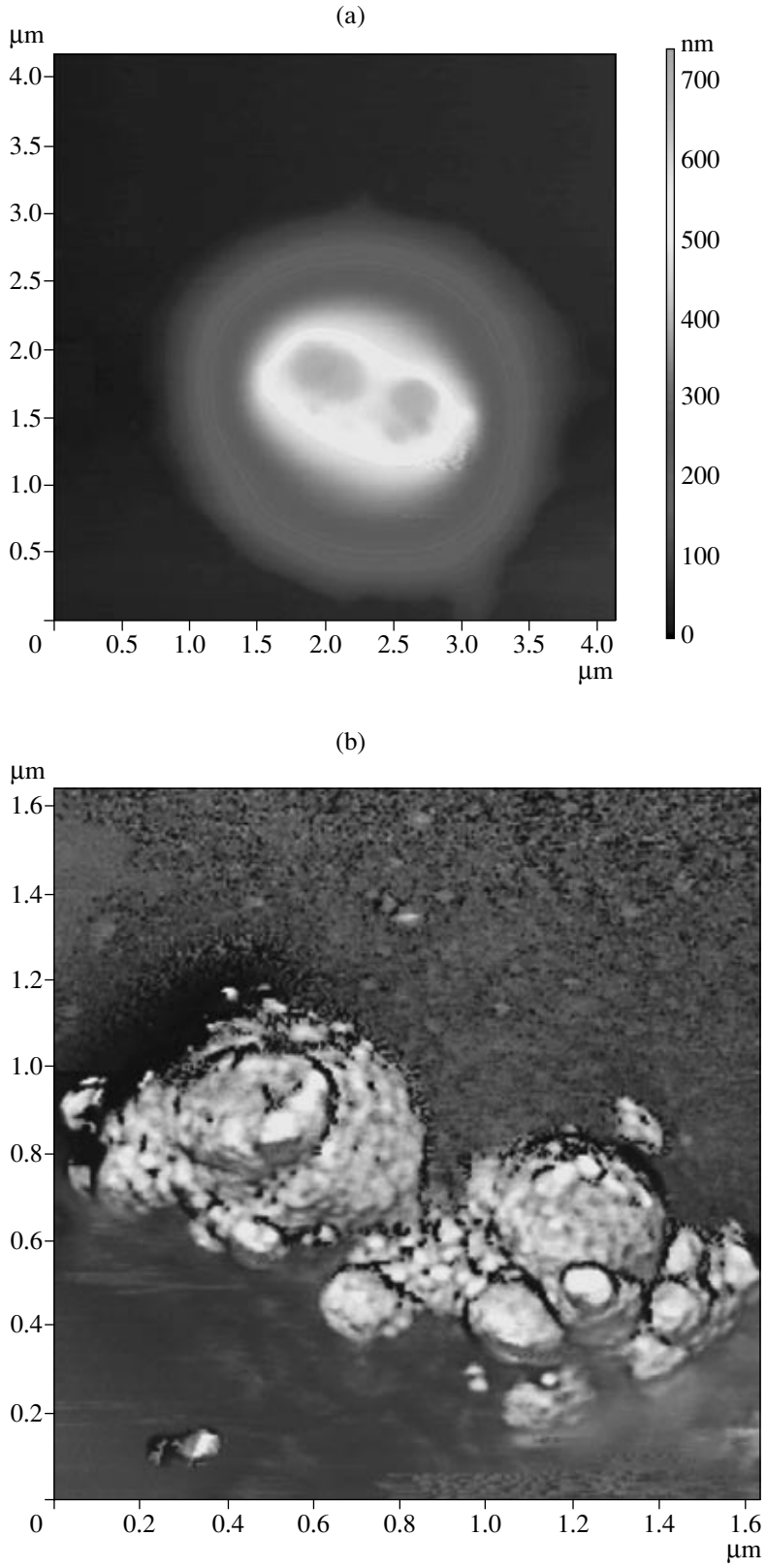

Fig. 1. (a) Topography of a single lipofuscin granule obtained with the use of an atomic force microscope (semi-contact mode). The image size is 4.1×4.1 μm; the diameter of the granule bottom, ~3 μm; the size of middle part of the granule, 1 μm; and the height, 700 nm. (b) Image of the central part of the granule obtained by the method of phase contrast. The image size is 1.6×1.6 μm. Two types of formations with characteristic sizes of 30–40 and 100–120 nm are seen.

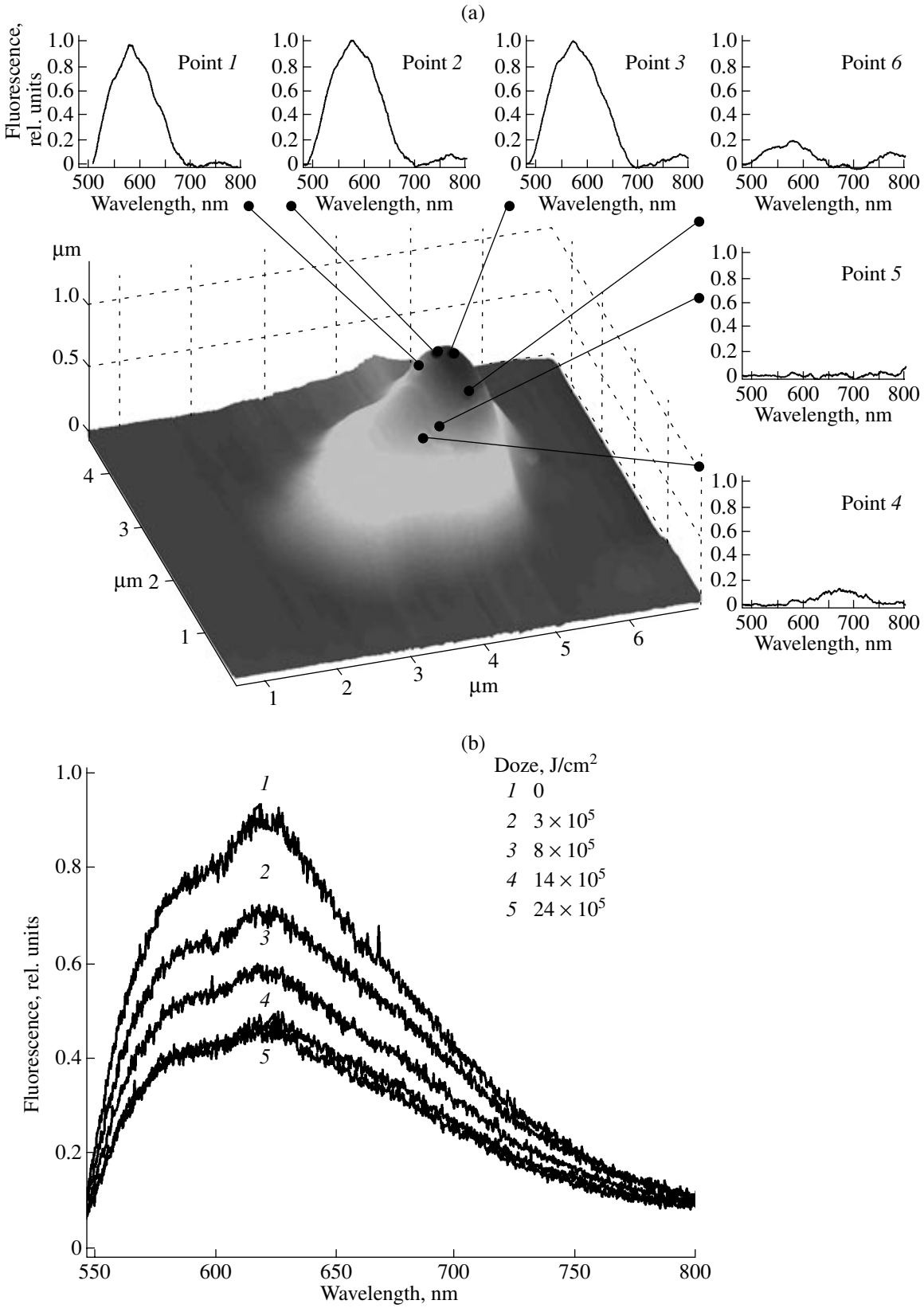

Fig. 2. (a) Topography of a single lipofuscin granule obtained with the use of a near-field microscope. The points in which we recorded the fluorescence spectra are marked. The respective fluorescence spectra are shown. (b) Decrease in the fluorescence intensity during long-term irradiation of a cluster of lipofuscin granules with light with a wavelength of 532 nm.

DISCUSSION

The data obtained with the use of electron microscopy suggest that the lipofuscin granule is homogeneous [8]. However, atomic force microscopy and near-field microscopy showed that it is not the fact. The granule is heterogeneous and consists of smaller aggregated particles. It is possible that these particles consist of "linked" insoluble proteins which, according to the data of chemical analysis, form granule by 30–70% [9]. Further ultrastructural, photo- and biochemical studies are required to understand the process of biological synthesis of lipofuscin granules in the RPE cells under normal and pathological conditions.

It is known that the excitation of lipofuscin granules with blue light induces fluorescence in the yellow range of spectrum ($\lambda_{max} \approx 600$ nm) [10]. It was hypothesized that this fluorescence is emitted by one of the main fluorophores (such as A2E and/or iso-A2E).

However, the study of single lipofuscin granules showed that the coincidence of the spectra of the granule fluorescence and the fluorescence of the fluorphore A2E does not mean that this fluorophore is the main fluorphore in all granules [11]. We found that, in addition to A2E, these granules contain several fluorophores which absorb light in the blue range of the spectrum. Moreover, it was hypothesized that yellow fluorescence of A2E is a result of energy transfer from other fluorophores.

We showed that the fluorescence of lipofuscin granule is heterogeneous. Not all granules fluoresced during excitation at 410 nm and, even in the fluorescing granule, fluorescence was detected only in single parts of the granule.

We found that, as a result of long-term irradiation of lipofuscin granule with visible light (wavelength, 532 nm), its fluorescence considerably decreased (Fig. 2b). This may mean that the fluorophore (or fluorophores) absorbing light in the range of 410 nm are destructed and converted into the products absorbing light in a more shortwave range of the spectrum. A change in the spectrum shape may be determined by the presence or appearance in the granule of other fluorophores which differ from A2E.

Indeed, in the experiments *in vitro* during irradiation of A2E with blue light we detected the appearance of several epoxy-forms of A2E (including nanoepoxides) and the main product, 7,8,7',8'-bis-epoxide [12]. It was recently shown that the main product of A2E photolysis is the relatively stable oxide of A2E, 5-8,5'-8-bis-furanoid [13].

An enhanced A2E synthesis and its conversion into oxidized products, epoxides, under the exposure to light was shown in the experiments *in vivo* with retina and pigment epithelium of knockout mice ($abcr^{-/-}$) which are characterized by an excessive accumulation of lipofuscin granules in RPE cells [14]. Note that the oxidation product of A2E (A2E monoepoxide) was found in the lipofuscin granule of pigment epithelium of human eye [15].

Bleaching of lipofuscin granule during irradiation with visible light and generation of new photooxidized products in the granule may be very important for the understanding of the mechanism of the intensifying effect of light on the development of degenerative deceases of retina.

ACKNOWLEDGMENTS

This study was supported by the Russian Foundation for Basic Research (project nos. 05-03-33199 and 02-04-49923) and the programs for fundamental studies of the Presidium of the Russian Academy of Sciences "Theoretical and Experimental Study of Nature of Chemical Bond and Mechanisms of the Most Important Chemical Reactions and Processes" (Program no. 1 OKhNM, Russian Academy of Sciences) and "Integrative Mechanisms of Regulation of Functions in Organism").

REFERENCES

1. Lamb, L.E. and Simon, J.D., *Photochem. Photobiol.,* 2004, vol. 79, no. 2, pp. 127–136.
2. Fishkin, N.E., Sparrow, J.R., Allikmets, R., and Nakanishi, K., *Proc. Natl. Acad. Sci. USA*, 2005, vol. 102, no. 20, pp. 7091–7096.
3. Ostrovskii, M.A., Dontsov, A.E., Sakina, N.L., *et al.*, *Sens. Sist.*, 1992, vol. 6, no. 3, pp. 51–54.
4. Boulton, M., Dontsov, A., Ostrovsky, M., *et al.*, *Photochem. Photobiol.*, 1993, vol. 19, pp. 201–204.
5. Rozanowska, M., Wassel, J., Boulton, M., *et al.*, *Free Rad. Biol. Med.*, 1998, vol. 24, pp. 1107–1112.
6. Kanofsky, J.R., Sima, P.D., and Richter, C., *Photochem. Photobiol.,* 2003, vol. 77, pp. 235–242.
7. Clancy, C.M.R., Krogmeier, J.R., Pawlak, A., *et al.*, *J. Phys. Chem. B*, 2000, vol. 104, pp. 12098–12100.
8. Schraermeyer, U. and Heimann, K., *Cell Res.,* 1999, vol. 12, no. 4, pp. 219–236.
9. Brunk, U.T. and Terman, A., *Free Rad. Biol. Med.*, 2002, vol. 33, pp. 611–619.
10. Delori, F.C., Dorey, C.K., Staurenghi, G., *et al.*, *Invest. Ophthalmol. Vis. Sci.*, 1995, vol. 36, pp. 718–729.
11. Haralampus-Grynaviski, N.M., Lamb, L.E., Clancy, C.M., *et al.*, *Proc Natl. Acad. Sci. USA*, 2003, vol. 100, no. 6, pp. 3179–3184.
12. Ben-Shabat, S., Itagaki, Y., Jockusch, S., *et al.*, *Angew. Chem., Int. Ed.*, 2002, vol. 41, pp. 814–817.
13. Dillon, J., Wang, Z., Avalle, L.B., and Gaillard, E.R., *Exp. Eye Res.*, 2004, vol. 79, no. 4, pp. 537–542.
14. Radu, R.A., Mata, N.L., Bagla, A., and Travis, G.H., *Proc. Natl. Acad. Sci. USA*, 2004, vol. 101, no. 16, pp. 5928–5933.
15. Avalle, L.B., Wang, Z., Dillon, J.P., and Gaillard, E.R., *Exp. Eye Res.*, 2004, vol. 78, no. 4, pp. 895–898.