Preparation of Bioadhesive Phosphorescent Particles and Their Use as Markers for Video-oculography of Mice

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Objective: To develop a bioadhesive phosphorescent particle that can be used as a marker in video-oculography to assess eye movements in the dark without drug treatment.
Methods: The marker was prepared by spray-coating a $Sr_4Al_{14}O_{25}$: Eu^{2+}, Dy^{3+} phosphor with a carboxyvinyl polymer. The morphologic, luminescent and adhesive properties were assessed. The dynamic properties of VOR measured by the marker were compared with those obtained by tracking the pupil under miotic treatment.
Results: Non-aggregated and non-fused particles having diameters of about 50 μm could be prepared by polymeric coating of the phosphor, resulting in particles small enough not to restrict eye movement. Although the phosphorescent of the particles decreased with increasing thickness of the coating layer, the coated particles were detectable in the dark for at least 60 min. The thicker the coating layer was, the higher the adhesiveness of the particles obtained. The particles having the thickest coating layer were retained on the corneal surface during VOR measurement and thus performed well as a marker in video-oculography. The dynamic properties of VOR measured by the marker were essentially identical to those obtained by tracking the pupil under miotic treatment.
Conclusion: Our marker will contribute to understanding the mechanisms underlying motor learning.

Key words: phosphorescence; bioadhesiveness; marker; video-oculography; vestibular-ocular reflex (VOR)

INTRODUCTION

Retinal image stabilization during self-motion is achieved via the combined operation of the vestibulo-ocular reflex (VOR) and the optokinetic reflex (OKR) [1]. The VOR is a reflex evoked by vestibular stimula and produces compensatory eye movement in the opposite direction to head motion. On the other hand, the OKR is elicited by image motion across the retina and generates eye movement in the same direction as visual motion. The neural circuits of the two reflexes are well defined, the sensory inputs can be artificially controlled, and the outputs, i.e. eye movements, can be quantitatively measured in laboratory animals and human [2]. For these reasons, the reflexes have been popular model systems to associate behavior with neurophysiology and to diagnose a variety of neurological disorders [3]. Recent advances in genetic engineering techniques in mice have made it possible to elucidate the function of a specific molecule at an individual level of animals [4]. Measurements of VOR and OKR in genetically modified mice are powerful tools for understanding the mechanisms underlying motor learning, which is the process by which one acquires the skills required for movements [5, 6].

The VOR is generally tested in the dark by sinusoidal oscillation of a turntable on which a mouse is mounted, whereas the OKR is measured in light by the sinusoidal oscillation about the vertical axis of a screen which surrounds the mouse [5, 6]. The magnetic search coil technique [7] and electro-oculography [8] were the major techniques used to trace the eye position of a mouse. Infrared video-oculography [9] gains wider acceptance due to its non-invasiveness and low cost. In this technique, an eye position is often determined from the pupillary center calculated from the eye image to analyze the two-dimensional eye movements. It is difficult to identify the pupillary center of a mouse in the dark, because the pupil becomes remarkably enlarged. Some kind of miotic, e.g. pilocarpine or hysostigmine, is often used to prevent pupillary dilation and to measure the VOR [10, 11]. Such cholinergic drugs and cholinesterase inhibiters have a pharmacological effect not only on the parasympathetic nervous system, such as miosis, but also on the central nervous system [12]. In fact, physostigmine has been used to improve the memory function of Alzheimer's patients [13]. The cholinergic activation of the nucleus reticularis pontis caudalis induces oculomotor phenomena that are somewhat similar to those described during REM sleep [14]. The use of drug that potentially affects the central nervous system should be avoided for VOR measurements of mice.

An alternative way is to use a marker placed on the

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cornea instead of the pupillar center to trace eye position. The marker should be biocompatible, detectable in the dark, and small enough so as not to restrict eye movement. Additional requirements are that the marker should remain in a fixed position on the corneal surface during measurement and be easily washed out after measurement. A small fluorescent marker was utilized in UV video-oculography more than 30 years ago by Batini *et al.* [15]. Currently available markers are titanium dioxide pigment used for tattoos and plastic-based fluorescent markers adhered with cyanoacrylate glue, and two approaches are generally considered as the 'gold standard' for measurement of three-dimensional eye rotation [16–19]. However, the markers have to be illuminated by light sources, e.g. black light, Wood's lamp and UV LED lamp, to detect in the dark. The UV exposure is undesirable, because it may harm the eye [20].

One method for solving the problem is development of light accumulating marker capable of emitting light in the dark. Phosphorescence is long-lasting luminescence, being different from fluorescence, and a number of phosphorescent materials have been widely studied for various device applications [21]. Strontium aluminates doped with the rare earth elements europium and dysprosium are one type of phosphor. Because of their excellent luminescent characteristics, such as high luminescent intensity, high quantum efficiency, long-lasting phosphorescence, and high chemical stability, $SrAl_2O_4 : Eu^{2+}$, Dy^{3+} and $Sr_4Al_{14}O_{25} : Eu^{2+}$, Dy^{3+} have drawn a great deal of interest from many researchers [22, 23]. These phosphors are kneaded with resins and molded into various devices, e.g. exit signs, traffic signs, and other safety-related signage. Using such phosphors, it may be possible to make a marker that is small but detectable in the dark without light source, for VOR measurements of mice.

Although the phosphorescent material itself does not adhere to the cornea, coating it with adhesive polymers can improve the adhesiveness of the material. Carboxyvinyl polymer, or poly (acrylic acid) is a water-soluble polymer which is popular as thickening, dispersing, suspending and emulsifying agents in pharmaceuticals and cosmetics [24]. The polymer has recently been employed to combine quantum dots with biological molecules in *in vivo* molecular and cellular imaging [25, 26]. It is expected that polymer coating gives the phosphorescent particles an ability to adhere to the corneal epithelial surface.

The aim of present study is to develop a bioadhesive phosphorescent particle that can be used as a marker in video-oculography. The particles were prepared by spray-coating a $Sr_4Al_{14}O_{25} : Eu^{2+}$, Dy^{3+} phosphor with a carboxyvinyl polymer. The morphology, size distribution, luminescence intensity, and adhesiveness of the coated particles were evaluated under various coating conditions to determine the most-suitable conditions for making markers for video-oculography. The dynamic properties of VOR in the dark were measured using the particle applied to the eyeball of a mouse, and the results were compared with those obtained by pupil tracking under miotic treatment.

MATERIALS AND METHODS

Preparation of bioadhesive phosphorescent particles

The preparation of bioadhesive phosphorescent particles was carried out by a spray-coating method [27]. LumiNova® BG series, BG-300M (Nemoto Lumi-Materials Co., LTD, Tokyo, Japan), which is a long afterglow $Sr_4Al_{14}O_{25}$: Eu, Dy phosphor, was used as a phosphorescent material. The excitation and emission peak wavelengths were 200–450 mm and 490 nm, respectively. The particle size range was 3–65 μm and the specific gravity was 3.9. The phosphor was sieved to collect particles with diameters of 32–45 μm. Ten grams of the particles were put into a truncated-conical coating pan (base diameter: 125 mm, top diameter: 105 mm, height: 125 mm), which was equipped with blades inside and made of stainless-steel. A carboxyvinyl polymer, Carbopol® 934 (Serva Electrophoresis GmbH, Heidelberg, Germany) was used as an adhesive polymer, and ethanol solutions of the polymer were prepared at concentrations of 0.5, 1.0 and 2.0% to coat the phosphor particles. The driblets (total 10 mL) of each ethanol solution were sprayed uniformly on the particles in the pan, which was rotated at 60 rpm. The coated particles were dried by warm air at 60°C. The coating procedure was repeated five or ten times, resulting in final particles.

Morphology and size analysis

The morphology of bioadhesive phosphorescent particles was imaged using a scanning electron microscope (SEM, S-3000N, Hitachi, Ltd., Japan). Before the SEM imaging, the polymer-coated or uncoated phosphorescent particles were placed on copper specimen supports and were coated with platinum using an ion sputterer (E-1010, Hitachi, Ltd., Japan).

The Martin diameter of each particle was calculated from the SEM image. Two-hundred particles were randomly selected, and the size distribution and volume mean diameter were determined for each type of phosphorescent particle.

Luminescent properties

Phosphorescent properties of polymer-coated or uncoated particles were characterized using a spectrofluorophotometer (RF-5300PC, Shimadzu Corporation, Kyoto, Japan). After storage in a darkroom for two days, the particles were irradiated by daylight-color, three-wavelength fluorescent light (FPL27EX-D, Panasonic, Osaka, Japan) at 2,000 Lux for 20 min. Immediately after that, the phosphorescent spectra and decay profiles of afterglow at 490 nm were measured without the excitation light.

Adhesive properties

The adhesiveness of the phosphorescent particles was evaluated using an *in vitro* model system, as shown in Fig. 1. Fifty milligrams of the particles were scattered uniformly on a phenol-formaldehyde resin plate (27 mm × 80 mm) sloped at 20° and were then washed out with an artificial lacrimal fluid, which consisted of 0.55% sodium chloride, 0.16% potassium chloride, 0.06% sodium carbonate, 0.18% dibasic sodi-

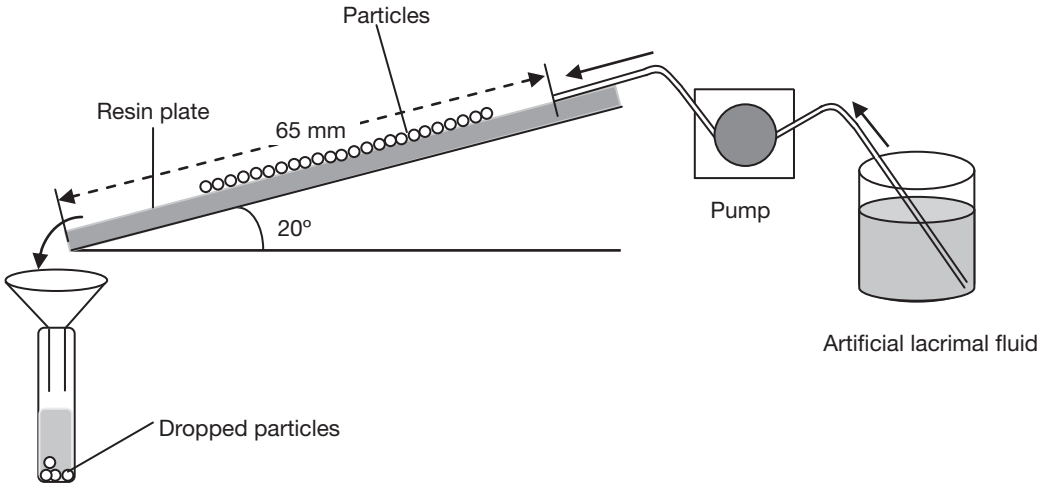

Fig. 1 Experimental setup for adhesiveness evaluation.

um phosphate hydrate, and 1.2% boric acid in distilled water, and were made to flow from the outlet into a test tube, which was set downstream, at 1.5 mL/min by a peristaltic pump (Model MS-Reglo, IDEX Health & Science GmbH, Germany). The particles washed out by artificial lacrimal fluids were collected at predetermined times and were rinsed with distilled water four times. After lyophilization by FreeZone® Freeze Dryers (Labconco, Kansas, USA), they were placed in a vacuum at room temperature for 1 day and then weighed. The percentage of particles remaining on the plate was calculated as an indicator of adhesiveness.

Animal preparation

Three C57BL/6 mice aged 8–18 months (body weights of 27–40 g) were used in the present study. All procedures for animal care and experimental protocols were carried out in accordance with the "Fundamental guidelines for proper conduct of animal experiments and related activities in academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology (2006)" and "Basic policies for the conduct of animal experimentation in the Ministry of Health, Labor and Welfare (2006)," and were reviewed and approved by The Institutional Animal Care and Use Committee at Tokai University.

All surgical procedures were performed under isoflurane anesthesia. After making a midline incision along the scalp, three stainless steel screws (Size 000, J. I. Morris, Southbridge, MA) were implanted in the cranial bone as anchors. A head post made of polycarbonate resin screw (M4 X 15, YAHATA, Nagoya, Japan) was cemented with dental acrylic (UNIFAST II, GC corporation, Tokyo) to the three anchor screws. The animal was allowed to recover for at least five days before measuring the eye movements.

Eye movement measurements

Each mouse was placed in a custom-made holder, in which its head post was immobilized and the whole body was loosely restrained by a vinyl chloride pipe (50 mm in diameter, 120 mm long). The animal holder was mounted on a turntable (60 cm in diameter) driven by a direct drive servo motor (NMR-CTFIA2A-841A, NIKKI DENSO, Kawasaki, Japan), which was controlled by a PC (Fig. 2). One drop (about 1 μL) of ophthalmic solution containing 3% pilocarpine hydrochloride (Sanpilo®, Santen, Osaka) was applied on the right eye of the mouse using a blunt needle (21G X 1 1/2", Terumo, Tokyo). After confirming miosis, a small area of the cornea was dried with the tip of a piece of filter paper and a bioadhesive phosphorescent particle was placed on this area as the marker. The horizontal VOR was induced by applying sinusoidal rotations, with an amplitude of 7.2° peak-to-peak, a peak velocity of 10°/s, and a frequency of 0.5 Hz, to the turntable. Frontal images of the right eye illuminated by an infrared LED lamp (M850L3, Thorlabs, Newton, NJ) were reflected by a hot mirror, monitored by an infrared-sensitive CCD camera (GV200, Library, Tokyo), and were stored as 640 x 480 pixel images at a sampling rate of 200 Hz in a PC.

The marker and pupil were tracked by an automated target tracking program (Move-tr/2D, Library). In the program, a stored eye image was transformed to a binary image, the center of the marker or pupil was estimated, and the x-position of the center was traced. The rotation angle was calibrated based on the x-position of each center, using the method of Sakatani and Isa [28]. The calculated angular velocity was then differentiated after filtering out noise at 10 Hz. The artifacts caused by saccadic eye motion and eye blinking were removed from the acceleration data by thresholding using Matlab (Mathworks, Natick, MA). The turntable velocity was determined with a sampling rate of 1000 Hz by a multi-channel continuous data acquisition and analysis package, Spike 2 (CED, Cambridge, UK). All velocity data were fitted to a sine curve using a nonlinear least squares regression program to estimate the gain (amplitude) and phase of the VOR. The VOR gain was calculated to be the ratio of the eye velocity amplitude to the head (turntable) velocity amplitude. The VOR phase is defined as the timing difference between the eye and the turntable velocity, and thus a value of zero represent the eye movement response required for a completely stable gaze, i.e. 180° out-of-phase to the turntable. The VOR gain and phase values for the marker and the pupil were compared. The signal to noise (S/N) ratios of marker and pupil

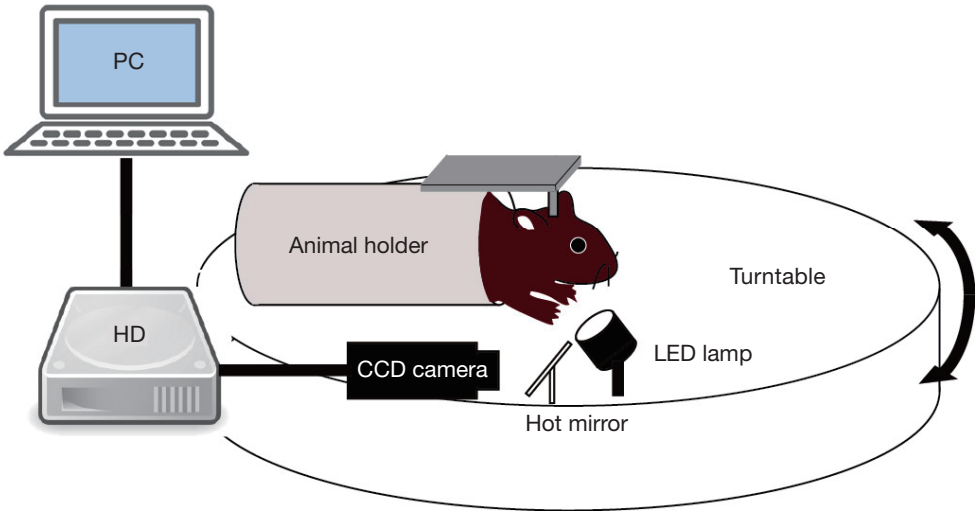

Fig. 2 Experimental setup for VOR measurement.

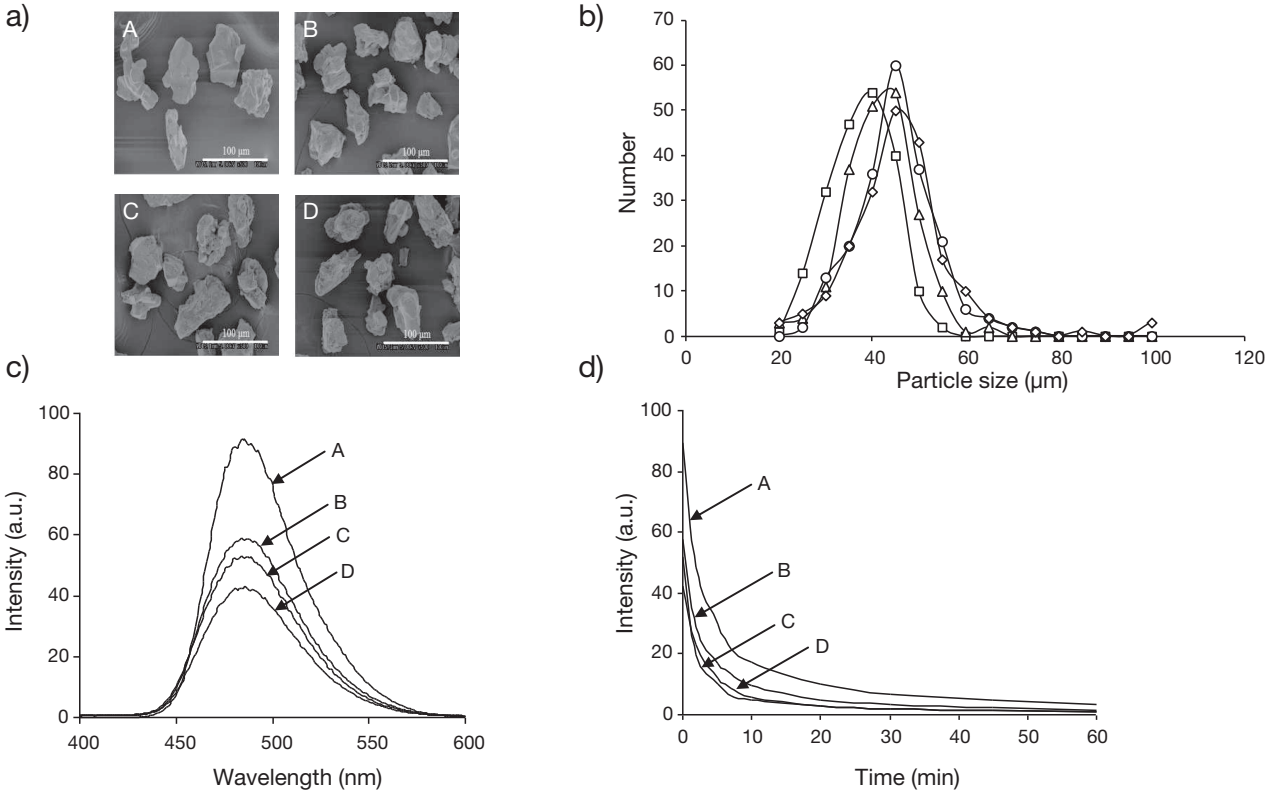

Fig. 3 Morphological characteristics and luminescent properties of phosphorescent particles. a) Scanning-electron-microscope images, b) size distribution, c) emission spectra and d) afterglow decay profiles of phosphorescent particles, which were uncoated (A, □), coated with 1.0% carboxyvinyl polymer solution 5 times (B, △), coated with 1.0% solution 10 times (C, ○), and coated with 2.0% solution 5 times (D, ◇). The size of particles was calculated from scanning-electron-microscope images. The luminescent properties were measured after UV irradiation for 20 min.

tracking were measured under no vestibular stimula. Statistical significance of difference was evaluated by paired t-test.

RESULTS

Morphological characteristics of bioadhesive phosphorescent particles

Fig. 3a shows scanning electron microscopic images of phosphorescent particles coated with carboxyvinyl polymer. The particles were prepared under various coating conditions, namely, coating with 1.0% carboxyvinyl polymer solution 5 times, coating with 1.0% solution 10 times, and coating with 2.0% solution 5 times. The particles were non-spherical, non-aggregated and non-fused, regardless of the polymeric coating conditions.

The size distribution of the particles is shown in Fig. 3b and the volume mean particle diameters are listed in Table 1. The polymeric coating increased the particle size, and the size increased with an increase in the

Table 1 Volume Mean Diameter of Phosphorescent Particles.

Phosphorescent particles	Volume mean diameter (μm)
Uncoated	42.62 ± 6.85
Coated with 1.0% polymer solution 5 times	46.94 ± 7.89
Coated with 1.0% polymer solution 10 times	50.75 ± 8.25
Coated with 2.0% polymer solution 5 times	54.41 ± 13.98

Each data represents the mean ± S.D. (n = 200).

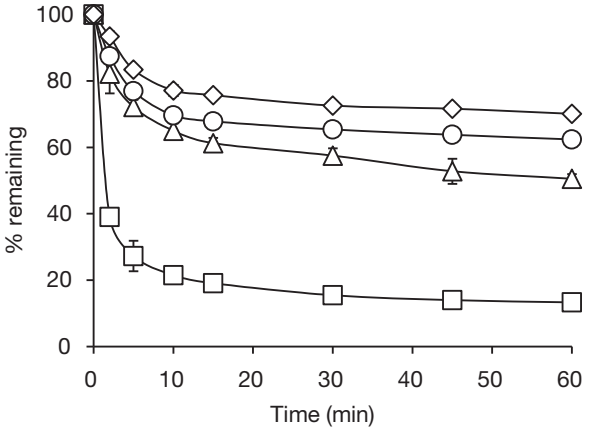

Fig. 4 Percentage of phosphorescent particles remaining on the phenol-formaldehyde resin plate after being washed with artificial lacrimal fluid. Uncoated (□), coated with 1.0% carboxyvinyl polymer solution 5 times (△), coated with 1.0% solution 10 times (○), and coated with 2.0% solution 5 times (◇).

polymer concentration of the spray solution and the number of spray-coatings. The diameters of all types of particles were around 50 μm.

Luminescent properties of bioadhesive phosphorescent particles

Fig. 3c shows the phosphorescence spectra of uncoated and coated particles. The data were recorded in the dark after 20 min irradiation with UV light. The spectrum of the uncoated particles exhibited a broad emission with a peak wavelength at 490 nm. The position of the emission peak in the phosphorescence was not changed by polymeric coating, whereas the emission intensity tended to decrease with an increase in polymer concentration and number of sprayings.

The decay profiles of afterglow at 490 nm are shown in Fig. 3d. All profiles showed a rapid decay and subsequent long-lasting phosphorescence, which reflects the long afterglow of $Sr_4Al_{14}O_{25}$: Eu, Dy phosphor. Emission intensities high enough to visualize the particles in the dark lasted for 60 min after removing the UV light, although the phosphorescence was weakened by polymeric coating.

Adhesiveness of phosphorescent particles

The adhesiveness of the phosphorescent particles was assessed by their adhesion to a phenol-formaldehyde resin. Uncoated phosphorescent particles fell off the resin plate immediately after starting to wash them out with the artificial lacrimal fluid and the percentage remaining was only 15 % after washing out for 60 min (Fig. 4). Coating the particles with carboxyvinyl polymer improved their adhesiveness to the plate, and the residue remaining on the plate increased depending on the polymer concentration and frequency of spray-coating. Biphasic profiles, in which a sudden drop was followed by a gradual decline, were observed for the coated particles as well as the uncoated ones.

Bioadhesive phosphorescent particles as a marker for VOR measurement

Small adhesive phosphorescent particles could be prepared, and their suitability as markers in video-oculography was then assessed by VOR measurements in the dark. Fig. 5a shows a screenshot of the mouse eyeball on which a phosphor particle coated with 2.0% carboxyvinyl polymer solution 5 times was applied. The pupillary constriction by miotic treatment was observed, and the pupil could be distinguished from the particle. The particle retained on the corneal surface during VOR measurement of at least 10 min.

Fig. 5b demonstrates an example of eye movement responses to sinusoidal oscillation of the turntable at 0.5 Hz with a peak velocity of 10°/s. The responses are represented as angular velocity data calculated from the position tracking of the pupil or particle. Compensatory eye movements, i.e. eye movements in the direction opposite to the head (turntable) movement for image stabilization, were observed. The time courses of eye movement velocity, including the artifacts caused by blinking, matched closely using both tracking methods. The VOR gain and phase obtained by marker and pupil tracking are listed in Table 2. There was no significant difference of VOR properties between two tracking methods ($p = 0.052$ for gain and $p = 0.945$ for phase). The S/N ratio of marker tracking ranged from 6.3 to 6.6 and that of pupil tracking was from 12.5 to 15.9.

DISCUSSION

Video-oculography with no drug treatment and no light source would be made possible by developing a

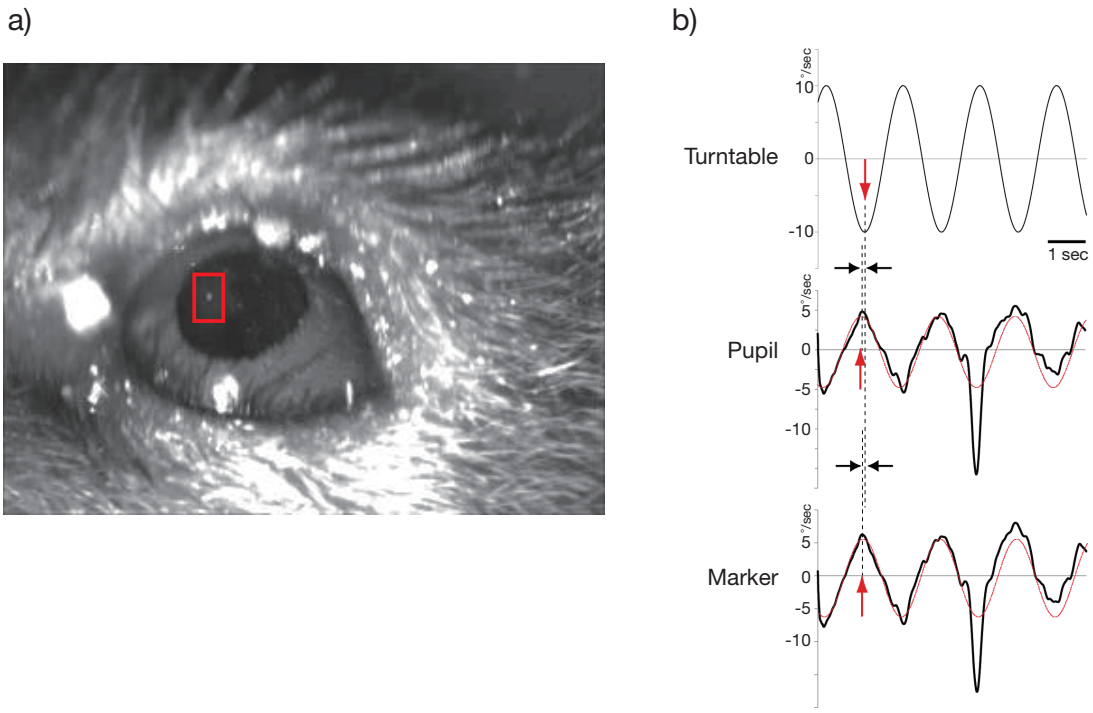

Fig. 5 In vivo validation of phosphorescent particles. a) Screenshot of bioadhesive phosphorescent particle on a mouse eyeball and b) examples of recorded VOR in mice. The particle was prepared by coating the phosphor with 2.0% carboxyvinyl polymer solution five times (shown in the red box). Eye movement velocities are obtained by applying sinusoidal rotations to the turntable at 0.5 Hz with a peak velocity of 10°/s, and are calculated from the position tracking of the pupil and marker. The red lines represent nonlinear least-squares fits of the data to the sine curve. The red and black arrows indicate times showing the peak velocity, and the timing difference between the eye and the turntable velocity, i.e. VOR phase.

Table 2 VOR Gain and Phase Obtained by Marker and Pupil Tracking.

	Marker	Pupil
Gain[a]	0.57 ± 0.06	0.64 ± 0.06
Phase (deg)[b]	23.8 ± 7.4	24.0 ± 7.9

Each data represents the mean ± S.D. (n = 8).
[a]No significant difference between two tracking methods (p = 0.052 by paired t-test).
[b]No significant difference between two tracking methods (p = 0.945 by paired t-test).

marker that is detectable by emitting light in the dark, that is small enough not to restrict eye movement, and that remains on the corneal surface during measurement. In the present study, we prepared such a marker by spray-coating a $Sr_4Al_{14}O_{25}:Eu^{2+}, Dy^{3+}$ phosphor with a carboxyvinyl polymer. The phosphor is one of the persistent luminescence materials providing typical emission bands in the blue–green region. The phosphor attracted our attention because of its good luminescent properties, such as long persistence time, high quantum yield, wide excitation range and high chemical stability [29]. Since the phosphor particle itself does not adhere to the cornea, we attempted to coat the particles with adhesive polymers. Carboxyvinyl polymer is popular as a pharmaceutical and cosmetic additive [24] and has recently been employed to combine quantum dots with biological molecules in *in vivo* molecular and cellular imaging [25, 26]. It is anticipated that the polymer can adhere to the corneal epithelial surface.

The size of bioadhesive phosphorescent particles was designed to be about 50 μm in diameter, considering the balance between the influence on eye movements and the detection sensitivity. The diameter was remarkably small compared that of eye balls (3.4 ± 0.3 mm, n=4). Since the small particle does not disturb the field of vision, it may be also useful for OKR measurement. The size was included in the size distribution of phosphorescent particles themselves (3–65 μm), so that only the sieving was required before the polymer coating. The resulting coated particles were not aggregated or fused (Fig. 3a), and the diameters were around 50 μm (Fig. 3b, Table I). There is a possibility that a small marker comes at the cost of less S/N ratio. Fortunately our marker was not the case (S/N ratio of 6.3–6.6).

The emission of the $Sr_4Al_{14}O_{25}:Eu^{2+}, Dy^{3+}$ phosphor is due to the d-f transition of Eu^{2+} [29, 30]. The fluorescence occurs when an orbital electron relaxes to its ground state by emitting a photon after being excited to a higher quantum state. On the other hand, the excitation of the molecule to its singlet excited state

is followed by intersystem crossing to a triplet state, which relaxes to the ground state by phosphorescence [21]. Our particles coated with carboxyvinyl polymer exhibited lower phosphorescence intensities than those of uncoated particles and the emission intensities decreased with an increase in thickness of the coating layer (Fig. 3c and 3d). However, the emission spectra were not affected by the coating (Fig. 3c). The afterglow of particles decayed in a biphasic fashion (Fig. 3d), in which the second long-lasting phosphorescence phase was mainly utilized to detect the particle in VOR measurements (Fig. 5b). The emission intensities were high enough to visualize the particles in the dark, with sufficient S/N ratio throughout the in vivo studies.

The carboxyvinyl polymer was selected as the coating material to make phosphorescent particles adhere to the corneal epithelium. It is known that an important mechanism of mucoadhesion is the swelling of the polymer [31]. In fact, the percentage of polymer-coated particles remaining on a phenol-formaldehyde resin plate subjected to washing with an artificial lacrimal fluid fell sharply for the first 10 min and thereafter decreased slowly (Fig. 4). This suggests that sufficient swelling and the accompanying adhesion take a certain time. The particle piled up on a particle may have been carried away without being able to swell enough for the first 10 min. A large amount of particles are needed in the in vitro adhesion test, which is a limitation of the test. In this study, the thickness of the polymeric coating was estimated to be 2.2–5.8 mm from the diameter data listed in Table I. The thicker the polymeric coating layer was, the more particles remained on the resin plate. The result also supports the reported adhesion mechanism related to the swelling of a polymer [32]. In adhesion to the corneal surface, the interpenetration of both polymer and glycoprotein chains across the biointerface may also play an important role in addition to the swelling [33]. The phosphorescent particle prepared by coating a phosphor with 2.0% carboxyvinyl polymer solution 5 times remained on the corneal surface during 10–60 min of VOR measurement (Fig. 5a). In this way, a marker which is detectable by emitting light in the dark, that is small enough not to restrict eye movement, and that remains on the corneal surface during measurement could be prepared.

The utility of our marker in video-oculography was appraised by VOR measurement of mice in the dark. VOR is a reflex evoked by head motion, namely vestibular stimula, which then produces compensatory eye movement in the opposite direction to head motion. On the other hand, OKR is elicited by motion of a visual image across the retina and produces eye movement to the direction same as the visual image. If the vestibular system is stimulated in a lighted environment, the observed response would become visually enhanced VOR [34]. The VOR measurement in total darkness should be enforced except in special circumstances. Infrared video-oculography is the most popular technique for tracking eye position in the dark in both clinical diagnosis and animal studies [3, 6, 9]. In most rodent studies, an eye position is measured by calculating the center of the pupil from the eye image and therefore some miotic treatment is necessary to avoid excess dilation of the pupil [10, 11]. In other cases, some kind of marker is used to trace eye position, but the requirement of UV light source is not appropriate for eye movement measurement [16, 17]. We thus tested whether the tracking system using our bioadhesive phosphorescent marker can be used in place of the conventional pupil tracking system. The time-dependent change in angular velocity of the eye could be described as a sine curve, which was almost 180° out-of-phase with the curve of the turntable (head). The pupil- and marker-derived eye movement records showed essentially identical results, and the velocity profiles were almost identical to each other, including the artifacts (Fig. 5b). The VOR gain and phase calculated using the marker were quite similar to the values calculated using the pupil (Table 2) and comparable to the previously reported values [9]. It means that the marker was retained on the cornea during measurements, and therefore marker tracking may be an alternative to pupil tracking. The present retention time (10–60 min) of marker on the cornea may be still insufficient. The particle on the cornea has been taken off when the mouse blinked hard. The thicker coating would improve the adhesiveness of particle, whereas it may make the detection sensitivity low. The use of other bioadhesive polymers such as chitosan is one of methods for the adhesive improvement. The phosphors can also be kneaded with resins and molded into a thin film, which may have a good balance of adhesiveness and emitting intensity. Further studies are necessary to develop a perfect marker.

For synchronous recording of the pupil and marker with an infrared-sensitive CCD camera, the mouse eye was illuminated by an infrared LED lamp and reflected by a hot mirror, which reflects infrared light and allows visible light to pass through. The wavelength of illumination light was 850 nm, which is considered to be outside the sensitivity range of mice retinas [35]. When the eye movement is measured by tracking only the marker, substituting for the pupil, both an infrared lamp and a hot mirror are unnecessary. Only a visible light camera and normal mirror, or no mirror, will be required, depending on the experimental setup for VOR measurement. A miotic has pharmacological effect on the central nervous system as well as the parasympathetic nervous system [20]. The miosis effect changes depending on time after drug administration and results in individual difference in mice. Since no miotic is used in our new method, one does not need to pay attention to side effects or differences of the miosis effect. On the contrary, the influence of miotic agents on the eye movements can be detected by our method from a comparison of profiles before and after drug treatment. If three markers are applied on the cornea and the x-y positions of each marker simultaneously are recorded, our technique can be extended to measure three-dimensional eye movements. The bioadhesive phosphorescent marker developed in the present study will also contribute to the wider application of such markers in the study of eye movements.

In this study, we prepared a bioadhesive phosphorescent marker used in video-oculography of mice by coating a $Sr_4Al_{14}O_{25}$: Eu^{2+}, Dy^{3+} phosphor with a carboxyvinyl polymer. The marker was detectable in the

dark, was small enough not to restrict eye movement, and stayed on the corneal surface during VOR measurement. The dynamic properties of eye movement measured by the marker were essentially identical to those obtained by tracking the pupil under miotic treatment. Our marker enables VOR measurement in mice without visual stimuli or drug treatment. This improved experimental technique will contribute to deeper understand of the mechanisms underlying motor learning, which is the process by which one acquires the skills required for movements.

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