Ultrasonic comparison of two morphologically distinct melanosomes in malignant melanomas[1]

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Summary. Ultrasonic measurement (0.333–200 MHz) of melanosomes isolated from B16 and Harding Passey (HP) mouse melanomas indicates that the partial wave resonance and principal relaxation of the 2 kinds of melanosomes are similar, but that their stochastic resonance is markedly different. The structure of the melanosomes appears basically amorphous, but linearly ordered and copolymeric in the molecular dimension of a segment composed of 5–6 zigzag units, which are packed closely in B16 and more openly in HP.

Malignant melanomas possess a unique biological property, i.e., synthesis of melanins and melanosomes. Even amelanotic melanoma produces melanosomes, though melanin synthesis within them is variable and usually scant as compared to melanotic melanoma. The melanosome is an unusually sophisticated energy conversion device capable of responding dynamically to its environment either by storing energy or converting energy from one form to another; this results in the production of cytotoxic substances[3–5]. Experimental data demonstrating that melanins and melanosomes are capable of absorbing ultrasound by an unusually efficient resonant transfer mechanism have largely come from studies employing synthetic melanins and sepia melanosomes[5–7] and, thus, little information has been obtained regarding natural mammalian melanins and melanosomes. To better understand the physico-chemical mechanisms which allow efficient energy transduction to melanosomes and which may be useful in providing a rational approach to treatment of melanoma, this study presents ultrasonic measurement of melanosomes isolated from B16 and Harding Passey (HP) mouse melanomas, and compares it with those of sepia melanosomes, the physical and chemical properties of which have been fairly well characterized[5–7]. B16 and HP melanomas are unique because they produce the 2 morphologically distinct melanosomes that are commonly seen in humans: B16 melanosomes, which are ellipsoidal-lamellar and HP melanosomes, which are spherical-granular.

Materials, methods and results. B16 and HP melanosomes were isolated and purified by our previously described method[8]. Briefly tumors, usually 30 g, were minced by scissors, layered on 0.5 M sucrose and spun to remove nuclear and large granule fragments. The supernatant above the 0.5 M sucrose was collected, and applied to a discontinuous sucrose density gradient ultracentrifugation (from 1.0 to 2.0 M sucrose, 105,000 × g for 2 h). The melanosomal fraction, obtained at the bottom, was then treated with a non-ionic detergent, 0.1% Brij 35 in 4 mM phosphate buffer, pH 6.8, to remove the outer membrane of the melanosomes. The purified melanosomes were dialyzed against distilled water, lyophilized, and used in the following experiments. Sepia melanosomes were collected from the ink sac of a cephalopod: *Sepia* (Acanthosepion) *sebaculecta* Sasaki. For ultrasonic measurement, the conventional technique[9,10] was employed using frequencies between 0.333 MHz and 200 MHz. Since sound absorption changes slowly after water is added to melanosomes and usually requires about 24 h to equilibrate, measurements were made a few days after the melanosomes were suspended. About 0.8 g of each type of melanosome was isolated. Suspensions of weight fractions of $C_w = 0.30$ in B16 and $C_w = 0.25$ in HP melanosomes were each held separately in an ultrasonic cell, i.e., a thin cylindrical cell, 2 cm in diameter and 0.08–0.2 cm thick, in which the 2 sides were joined together by a thin film of polyvinylidene chloride (~ 0.02 mm). The longitudinal absorption coefficient in the diluted suspension is expressed as

$$\alpha = \alpha_1 + \alpha' \qquad [1]$$

where α_1 and α' are the attenuation coefficients of water and of the melanosome, respectively[11–13]. It was found that absorptions below 100 MHz, referred to as scattering and viscous drag effect, were negligibly small. The ratio of α' to the frequency of sound f is expressed as

$$\alpha'/f = \pi V K'' C/K'^2 \qquad [2]$$

where V is the sound velocity in suspension, C is the volume fraction of the melanosome in water, and K′ and K″ are, respectively, the real and imaginary parts of the bulk modulus of the melanosome.

The absorption coefficients per frequency for the suspensions at 23 °C are plotted against frequency in the figure, with 3 peaks of around 1 MHz, 30 MHz and 200 MHz being shown. It may be seen that the curves for B16 and HP melanosomes are quite similar to that of the sepia melanosomes shown by a chain line, with the exception of the lowest frequency region in B16 melanosomes, where the magnitude of absorption at 1.25 MHz had a factor 30 times smaller than that of HP melanosomes. Since the value of α_1/f^2 was 208×10^{-17} db cm^{-1} sec^2 at 23 °C and 400×10^{-17} at 3 °C between frequencies of 100 MHz and 310 MHz, α_1 was neglected in the equation (Eq) [1]. To relate these peaks to the molecular motion involved, we may refer to previous data[5–7,13], where the spectra of sepia melanosomes have been interpreted using 3 terms: 1. resonance at 95 MHz referred to as stacking of stiff chains due to the standing mode of particle waves, 2. primary relaxation defined by the single relaxation spectrum found in linear chain polymers and associated liquids and ascribed to the cooperative molecular motion of the backbone chain or to the breaking and reforming of molecules in a cluster, and 3. stochastic resonance in an anomalously large absorption to describe non-equilibrium and irreversible properties of thermodynamics in a living system. Terms 1 and 3 are expressed as the resonant equations

$$K' = K_0 + (1/K_{r0} - 1/\omega^2 K_{r2})[(1/K_{r0} - 1/\omega^2 K_{r2})^2 + (1/\omega K_{rl})^2]^{-1} \qquad [3a]$$

and

$$K'' = (1/\omega K_{rl})[(1/K_{r0} - 1/\omega^2 K_{r2})^2 + (1/\omega K_{rl})^2]^{-1} \qquad [3b]$$

where K_0 is the limiting bulk nodules at very low frequencies, K_{r0} is the elastic constant, K_{rl} is the viscosity, and K_{r2} is the inertia term. The Q value is expressed as $Q = K_{rl}/\omega_r K_{r2}$ for the resonant frequency of $2\pi f_r = \omega_r = (K_{r0}/K_{r2})^{1/2}$. Using the parameters listed in the table Eq [1] is shown as a dashed line in the figure.

Term 2 is expressed as the resonant equation of

$$K'' = K_{r0}\omega\tau(1 + \omega^2\tau^2)^{-1} \qquad [4]$$

with a single relaxation time of $\tau = (2\pi f_r)^{-1}$. B16 melanosomes at 1.25 MHz and at about 30 MHz follow Eq [4]. Likewise, HP melanosomes follow Eq [4] at 30 MHz, indicating that the primary relaxation of B16 and HP melanosomes is similar.

The Q values for $\gamma \gg 1$ is approximately related to the forms of $Q = (\gamma/2\pi)\,(q' - q)\,(q' + q)^{-1}$ and

$$q = (q'/\gamma)\exp(-\varepsilon/KT) \qquad [5]$$

where γ is the number of clusters around the void whose energy exceeds that of the state of clusters by ε, which is the activation energy for hopping conduction with respect to electrons; k is the Boltzmann constant, T is the absolute temperature, and q and q′ are the forward and backward transition rates with regards to phonon-electron interaction, respectively[14]. Roughly speaking when q is far less than q′, the γ is expressed as $\gamma = 2\pi Q$.

Parameters of relaxation and stochastic resonance for suspension of B16, HP and sepia melanosomes at 23 °C

Substance	f_r (MHz)	K_0 (dyne/cm^2)	K_{r0} (dyne/cm^2)	K_{r1} (P)	K_{r2} (g/cm)	Q
B16 in 70 wt % water	1.25		1.27×10^{10} [a]			0.2
HP in 75 wt % water	0.35	$\sim 1.3 \times 10^{10}$	2.68×10^{10}	1.37×10^{4}	5.55×10^{-3}	1.1
Sepia in 60 wt % water	0.38	1.5×10^{10}	2.2×10^{10}	1.27×10^{4}	3.98×10^{-3}	1.4

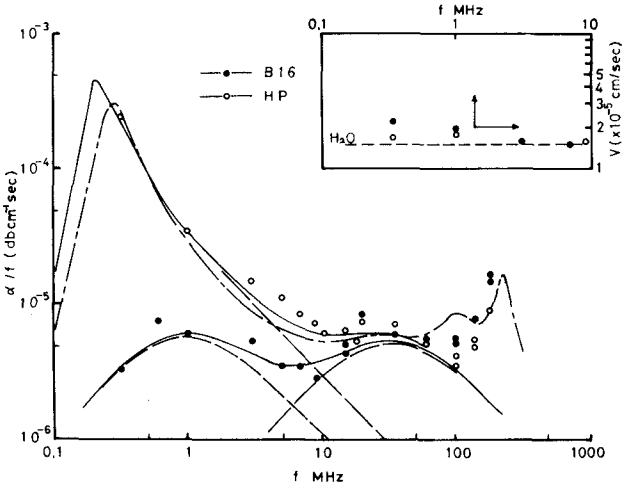
[a] Estimation from $K''_{max} = K_{r0}/2$.

The measured Q of the HP melanosomes was resulted in $\gamma = 6.9$, which is comparable to that of sepia melanosomes, while the measured Q of the B16 melanosomes was resulted in $\gamma = 1.3$. This difference may be large enough to cause hopping conduction of the melanosome, which is strongly related to its cytoprotective function within the cell in energy conversion and transduction[15]. Since the biophysical reaction of electron transfer is an essentially irreversible process in in vivo biological systems, the peculiar reaction form of Eq [5] that developed in HP melanosomes is rather reasonable. On the other hand, the low absorption of B16 melanosomes was close to a single relaxation, indicating an equilibrium process in a dead system with a usual equation of $q = q' \exp(-\varepsilon/KT)$.

If term 2 is attributed to principal relaxation, f at the peak of α/f_r should shift to low frequency with a decrease in temperature following the rate process. Curiously, however, no appreciable shift was observed. Thus, the activation energy for diffusion of B16 and HP melanosomes may be less than that of sepia melanosomes. Above 100 MHz, α'/f sharply increased with increasing frequency, suggesting the presence of term 1. The rise of absorption in B16 and HP melanosomes was relatively steep, indicating an ordered structure of the 2 melanosomes with high Q.

Discussion. The present study has led to the following 3 basic conclusions with respect to the physicochemical structure of the melanosomes in B16 and HP melanomas. First, the partial wave resonance observed above 100 MHz (term 1), reflecting molecular motion in the local order, indicates that the structure of both B16 and HP melanosomes is linearly ordered. Second, the similarity in principal relaxation (term 2) between B16 and HP melanosomes shows that the conformational structure of the 2 melanosomes is basically amorphous and copolymeric in the molecular dimension of a segment composed of 5–6 zigzag units. This is in marked contrast to the network structure with a permanent covalent binding between neighboring molecules proposed in synthetic melanins[16]. Third, and most importantly, the marked difference in stochastic resonance (term 3) between B16 and HP melanosomes indicates that the structure of B16 melanosomes is closely packed while that of HP melanosomes is more open, similar to that of sepia melanosomes. This difference probably reflects the different morphology of the 2 melanosomes. In this regard, it is interesting to note that the fine structure of both HP and sepia melanosomes is spherical and granular.

Furthermore, the significant absorption of HP-type melanosomes at 1 MHz suggests that ultrasound should prove an effective modality for malignant melanoma, because, as the sound wave travels through the melanosome, sound energy is transferred to and lost in the melanosome at a rate of $2\alpha' I = H$, where I is the intensity of the wave and H is heat production, causing local heat around the melanosome. Previously, ultrasound, because of its advantage for localized deep heating, either alone or in combination such as with X-rays, has been used to achieve hpyerthermia in various malignant tumors[17–19]. It may now, however, also provide a potential, specific new modality for certain types of malignant melanoma, e.g., those melanomas producing HP-type melanosomes, by synergism of local hyperthermia and cytotoxicity through efficient energy transduction from the melanosomes.



Sound absorption and velocity of B16 and HP melanosomes in suspension plotted against frequency. Solid lines represent composite behavior based upon Eqs. [3] and [4].

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