A Nanodiamond-peptide Bioconjugate for Fluorescence and ODMR Microscopy of a Single Actin Filament

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Recently, the importance of conformational changes in actin filaments induced by mechanical stimulation of a cell has been increasingly recognized, especially in terms of mechanobiology. Despite its fundamental importance, however, long-term observation of a single actin filament by fluorescent microscopy has been difficult because of the low photostability of traditional fluorescent molecules. This paper reports a novel molecular labeling system for actin filaments using fluorescent nanodiamond (ND) particles harboring nitrogen-vacancy centers; ND has flexible chemical modifiability, extremely high photostability and biocompatibility, and provides a variety of physical information quantitatively via optically detected magnetic resonance (ODMR) measurements. We performed the chemical surface modification of an ND with the actin filament-specific binding peptide Lifeact and observed colocalization of pure Lifeact-modified ND and actin filaments by the ODMR selective imaging protocol, suggesting the capability of long-term observation and quantitative analysis of a single molecule by using an ND particle.

Keywords Actin filament, nanodiamond, bioimaging, single molecular observation

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Introduction

Living cells are continuously exposed to the external mechanical forces applied by the adjacent cells and extracellular matrix. The external forces are sensed by the cell presumably through conformational changes in various elements, for example, α-catenin, mechanosensitive channels, and Cas protein, which become transduced into intracellular biochemical signals of protein-protein interaction, ion conduction, and phosphorylation, respectively. Such signal transduction processes are known as mechanotransduction. Recently, mechanical forces have been proposed to change the function of an actin filament, which is a major cytoskeletal component in maintaining the structural integrity of a cell. It has been reported that coflin interacts selectively with a tensionless actin stress fiber in contrast, the affinity between an actin filament and myosin II can be enhanced by stretching the actin filament. Thus, actin filaments are considered to change conformations dynamically and play critical roles in mechanotransduction. However, the conformational changes in a single actin filament induced by external forces have, to date, not been adequately observed and analyzed because of the lack of suitable methods for the reliable long-term single-molecule measurement.

Fluorescent probes are generally used to visualize the target protein in a living cell. As a major use of the probes, they can be conjugated with a protein or peptide that specifically recognizes the target protein. Therefore, the location of the target protein can be detected from the fluorescence of the probe. In particular, fluorescent dyes, fluorescent proteins, and quantum dots are known as representative probes. However, visualization using fluorescent dyes or fluorescent proteins does not enable observation of fluorescence for a period of minutes because of their low photostability. In addition, the observation using quantum dots is difficult because they have low biocompatibility. Although such probes are suitable for detecting the location of the target protein or organelle, in terms of photostability and cytotoxicity, it is difficult to detect the structural change or motion of a protein requiring long-term observation.

The photostability of fluorescent nanodiamonds (NDs) allows for long-term fluorescence observation for hours and an accurate measurement of the photon emission. Due to this special
property, NDs have emerged as a potential probe for single-molecule fluorescence imaging. Moreover, the stable fluorescence is closely coupled with the spin state of electrons in the nitrogen-vacancy color center (NVC) of the ND; therefore, the spin state of the NVC can be determined by measuring the fluorescence intensity and provides detailed information regarding the environment such as magnetic field around the probe. Furthermore, the difference in the fluorescence intensities between spin states of NVC enables exclusion of the intrinsic fluorescence of most biological samples, which hinders single-molecule observation, to obtain the selective image of the ND alone through an ODMR measurement technique called selective imaging protocol (SIP). For these reasons, we considered that NDs are novel and one of the most feasible analytical materials for measuring a single actin filament.

Here, we show a simple and effective method for labeling a single actin filament with NDs based on the interaction between actin and an actin-binding peptide Lifeact. Lifeact, which is the short peptide of the first 17 amino acids from the yeast protein Abp 140, is a specific targeting tag for visualizing an actin filament in a living cell. Another common approach for visualizing an actin filament is the injection of phalloidin, which originates from Amanita phalloides mushroom and is a small molecule specifically bound to an actin filament. However, phalloidin paralyzes the cytoskeleton and leads to cell death because it irreversibly binds the actin filament. On the other hand, Lifeact is more convenient because not only does it not compete with other actin binding proteins, but potentially it also does not affect the dynamics of actin filaments in contrast to the phalloidin objective (ApoTIRF, NA = 1.49, Nikon) and an EMCCD camera (DU-860, Andor Technology) was used for all fluorescence and ODMR measurements. A blue solid-state laser (488 nm, Sapphire 488 LP, Coherent) and a green solid-state laser (532 nm, Sapphire 532 LP, Coherent) were used for fluorescence excitation. A synthesizer (E8257D, Agilent) was used for spin excitation. FluoroMax-4 spectrofluorometer (Horiba Scientific) was used for fluorescence measurements to determine the amount of fluoresceinamine on ND surface.

**Apparatus**

An OptimaTM TLX Ultracentrifuge (C7X11A07, Beckman Coulter) was used for actin purification. An inverted microscope (Eclipse Ti, Nikon) equipped with an oil-immersion ×60 objective (ApoTIRF, NA = 1.49, Nikon) and an EMCCD camera (DU-860, Andor Technology) was used for all fluorescence and ODMR measurements. A blue solid-state laser (488 nm, Sapphire 488 LP, Coherent) and a green solid-state laser (532 nm, Sapphire 532 LP, Coherent) were used for fluorescence excitation. A synthesizer (E8257D, Agilent) was used for spin excitation. FluoroMax-4 spectrofluorometer (Horiba Scientific) was used for fluorescence measurements to determine the amount of fluoresceinamine on ND surface.

**Actin purification**

In a cold room at 4°C, we soaked acetone powder extracted from rabbit muscle was donated by Prof. Harada, Kyoto University. G-buffer for actin purification consisted of 2 mM Tris(hydroxymethyl)aminomethane (Tris-HCl, 35434-21) at pH 8.0, 0.2 mM ATP (adenosine triphosphate) and 0.1 mM CaCl₂. F-buffer for actin polymerization consisted of 250 mM KCl, 5 mM MgCl₂, 50 mM imidazole at pH 7.0 and 2.5 mM EGTA at pH 8.0. Tris-HCl and DTT (dithiothreitol, 14112-52) were purchased from Nacalai Tesque. Adenosine 5'-triphosphate disodium salt hydrate (ATP, A7699-1G) was purchased from Sigma-Aldrich. CaCl₂ (calcium chloride, 038-07385), KCl (potassium chloride, 163-03545), MgCl₂ (magnesium chloride, 136-03995), NaN₃ (sodium azide, 195-11092) and imidazole (905-00015) were purchased from Wako Pure Chemical Ind. EGTA (ethylene glycol tetraacetic acid; GEDTA, 346-01312) was purchased from Dojindo Laboratories. Rhodamine-phalloidin (PHDR1) for staining F-actin was purchased from Cytoskeleton, Inc. ND (MD30) was purchased from Tomei Diamond Co. Ltd. Pyridine (166-05316), ND (MICRON+ MDA M0.10) was purchased from Element Six, while succinic anhydride (194-04352) and NHS (N-hydroxysuccinimide, 089-04032) were purchased from Wako Pure Chemical Ind. EDC (1-ethyl-3-(3-dimethyaminopropyl) carbodiimide, hydrochloride, 348-03631) was purchased from Dojindo Laboratories. N-(2-Aminoethyl) maleimide hydrochloride (A2436) was purchased from TCI. Fluoresceinamine, isomer I for labeling ND was purchased from Sigma-Aldrich. Lifeact peptide (MGVADLIKKFESISKEEWGGSC) was synthesized by and purchased from Toray Research Center.

**Experimental**

**Reagents and chemicals**

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During the dialysis, the buffer was exchanged three times. Three days later, we centrifuged (2°C, 54,400g, 15 min) to remove the actin filament losing the depolymerizing ability, and as a result, G-actin was purified. To prepare the observation sample, we added 1 ml of F-buffer to polymerize G-actin, and then, adjusted the solution to 1 μM of G-buffer. Next, we added rhodamine-phalloidin into the solution containing the actin filament, and then, finished labeling the actin filament with rhodamine-phalloidin (rhodamine-F-actin).

**Lifeact-modified nanodiamond (ND-Lifeact)**

COOH-functionalized nanodiamonds (ND-COOH) were prepared as previously reported. In addition, we modified the ND-COOH surface with hyperbranched polyglycerol (ND-HPG) as previously reported, in order to suppress nonspecific adsorption to the protein and obtain high dispersibility. Next, we suspended 2.7 mg of ND-HPG with 2 ml of pyridine, and added 39.8 μl of 0.5 mM succinic anhydride in pyridine to the ND-HPG solution. We then stirred it at 70°C for 1 h under argon to functionalize the surface by COOH groups (Scheme 1A). After the reaction, we centrifuged (Beckman 10°C, 87,100g, 1 h) to remove the ND-HPG-COOH by DLS size measurement. These were fitted with semilogarithmic Gaussian distribution as indicated with red and blue solid lines, respectively.

**Scheme 1** Schematic procedure for the preparation of ND-Lifeact. (A) COOH functionalization of OH group with HPG. (B) NHS functionalization of COOH group with ND-HPG-COOH. (C) Surface modification of ND-HPG-NHS with fluorescein and maleimide at NHS. (D) Michael addition reaction between cysteine of Lifeact and maleimide group on ND to conjugate Lifeact with ND surface.

**Fig. 2** Characterization of ND-Lifeact. (A) FT-IR spectra comparison of ND-HPG-COOH (blue), Lifeact (green) and ND-Lifeact (red). A dashed line at approximately 1650 cm⁻¹ shows the characteristic absorption of Lifeact. (B) Hydrated-diameter distribution of Lifeact conjugated (red) and nonconjugated (blue) ND-HPG by DLS size measurement. These were fitted with semilogarithmic Gaussian distribution as indicated with red and blue solid lines, respectively.
property but also electron spin resonance (ESR) analysis was performed, indicating that no less than a hundred NVCs in NDs obtained using SIP. An ND particle (indicated by the red arrow) was used for acquiring the ODMR spectrum in Fig. 4. (E) Merged images of the rhodamine-F-actin (A) and NVCs in ND (D). NVCs were also colocalized with rhodamine-F-actin.

and then obtained ND-HPG(fluorescein)-maleimide (Scheme 1C). The obtained ND-HPG(fluorescein)-maleimide was suspended in 200 μl of Milli-Q water, mixed with 1.2 mg of Lifeact in 800 μl of Milli-Q water, and stirred for 2 h (Scheme 1D). We centrifuged (10°C, 87100g, 1 h) the mixture and then suspended the pellet with Milli-Q water. We repeated this procedure twice to obtain ND-Lifeact.

To confirm the synthesis of ND-Lifeact, we measured FT-IR spectra of ND-HPG-COOH, Lifeact and ND-Lifeact. As a result, a characteristic absorption peak for Lifeact (Fig. 2A, green) approximately at 1650 cm$^{-1}$ (indicated with dashed line), which was not found in the spectrum of ND-HPG-COOH (Fig. 2A, blue), was observed in the spectra of ND-Lifeact (Fig. 2A, red), instead of a sharp and strong absorption of C=O stretching at 1731 cm$^{-1}$ in the spectra of ND-HPG-COOH. To evaluate the reaction efficiency of fluoresceinamine with NHS on the surface of ND-HPG-NHS, quantitative fluorescence analysis was performed, indicating that no less than a hundred fluorescein molecules were bound to the surface of a particle although the efficiency was relatively poor. Additionally, we performed DLS size measurements to measure hydrated diameters of ND-HPG before and after Lifeact conjugation (Fig. 2B, blue and red, respectively), and results showed that the hydrated diameters (mean ± standard deviation) were 50.2 ± 17.0 and 118.6 ± 54.1 nm, respectively. It is reasonable to suppose that the particle size of the ND-HPG was enlarged by the addition of Lifeact peptide chains. Accordingly to the results of FT-IR and DLS measurements, we conclude that the series of conjugation reactions in Scheme 1 was carried out between ND and Lifeact, and thus ND-Lifeact conjugate was successfully obtained.

Principle of ODMR and procedure of ODMR-based SIP

An NVC harbors a pair of electrons in stable triplet state under ambient conditions, which has not only fluorescence property but also electron spin resonance (ESR) activity corresponding to the triplet spin quantum number $S = 1.12$. Additionally, the fluorescence intensity of NVC in a spin state of spin-magnetic quantum number $M_s = \pm 1$ is different from that of $M_s = 0$, because the relaxation pathways of the photo-excited NVC electron pair depend on the spin state; therefore the magnitude of NVC magnetic resonance can be measured indirectly via fluorescence intensity in ODMR measurement. Moreover, photo-excited NVC in $M_s = \pm 1$ relaxes selectively to $M_s = 0$ of ground state through a triplet-singlet intersystem crossing; the results is that the electron spins in NVC can easily be pumped from poorly polarized Boltzmann population distribution to highly polarized (>0.7) spin state. This nature of NVC enables ultra-sensitive ODMR measurements even of a single NVC.

ODMR-based SIP previously reported is a high-contrast imaging method to find NVCs selectively by fluorescence measurement. The fluorescence intensity of NVC can be modulated onto a carrier frequency of ESR-excitation microwave. Thus a series of the modulated fluorescence images containing bright spots of NVC can be reconstructed into a selective image of NVCs by lock-in detection. SIP allows reliable measurement even of a single molecule labeled with an ND, because we can find out the fluorescence signal of NVC in the ND distinctively from other signals such as autofluorescence of biological samples. An overlap of fluorescence excitation wavelength spectra between rhodamine-phalloidin and NVC was so large that we could not distinguish the fluorescence signals of them clearly by commonly used fluorescence microscopy. Thus we extracted the signal of NVCs from the mixed signal and reconstructed SIP images indicating locations of ND by ODMR-based SIP.

Observation of rhodamine-F-actin labeled with ND-Lifeact

ND-Lifeact was mixed and interacted with rhodamine-F-actin for 1 h. We dropped the mixture on a coverslip and covered it with another coverslip. The sample was observed using fluorescence and ODMR-based SIP by an inverted microscope (ECLIPSE Ti, Nikon) equipped with an oil-immersion 60× objective (ApoTIRF, NA = 1.49, Nikon) and an EMCCD camera (DU-860, Andor Technology). Fluorescein was excited with a blue solid-state laser (488 nm, Sapphire 488 LP, Coherent). Rhodamine-phalloidin and NVCs in the ND were excited with a green solid-state laser (532 nm, Sapphire 488 LP, Coherent). The fluorescence data of fluorescein, rhodamine-phalloidin, and NVCs in the ND were collected through 540 – 550 and 575 – 625 nm bandpass filters and a 635-nm long-pass filter, respectively. ODMR-based measurements were performed under microwave irradiation at 2.87 GHz generated by a synthesizer (E8257D, Agilent) for exciting electron spins in NVCs.

Results and Discussion

We observed actin filaments labeled with rhodamine-phalloidin at the single filament level by fluorescence observation...
(Fig. 3A). The fluorescence of fluoresceinamine bound to ND-Lifeact could also be observed (Fig. 3B), and was colocalized with the rhodamine-phalloidin bound to the actin filament (Fig. 3C). Based on the ODMR signal reconstructed from the NVC as an image using SIP, the fluorescence bright spots of the NVC in ND-Lifeact were observed by separation from the nearly same-color fluorescent rhodamine (Fig. 3D), and were also colocalized with the rhodamine-phalloidin bound to the actin filament (Fig. 3E). Note that the bright spots of the NVC were colocaled with small parts of the actin filament, contrary to the result observed with fluorescein. However, these results also reasonably agreed with the fact that NDs treated with neither electron- nor ion-irradiation rarely contain NVCs. In contrast, no colocalizations of non-Lifeact-modified ND with an actin filament labeled with rhodamine-phalloidin were observed. In addition, the NVCs in ND-Lifeact bound to the actin filament allowed us to obtain ODMR spectra (Fig. 4), which are known to provide a variety of physical information regarding the environment around the ND as electron spin resonance frequency. These results were reproducibly obtained in independent multiple experiments. Therefore, we confirmed that it is possible to label a single actin filament with ND-Lifeact.

One of the other advantages of ND-Lifeact is extraordinary photostability. Especially, NVC has superior photobleaching resistance to the other fluorescence dyes such as rhodamine. Figures 5A – 5D shows typical time-series images of rhodamine-phalloidin-stained actin filaments labeled with ND-Lifeacts, which were continuously exposed to a 3-mW green laser for 20.48 s. As a result, the rhodamine fluorescence was almost completely photobleached after about 7.68 s of laser exposure. In contrast, fluorescence bright spots of ND-Lifeact remained after 10.24 s of laser exposure, and allowed reconstruction of a high-contrast SIP image of the ND as shown in Fig. 5E.

NVC is a photon source emitting stable photons for several hours because of its extremely high photostability (Fig. S1, Supporting Information). Therefore, we might observe the dynamic structure of an actin filament from the fluorescence over long periods. In the biological phenomena affected by mechanotransduction and mechanical stimulation, differentiation, cell migration, and apoptosis take several hours or several days. In addition, it is well known that such phenomena are directly or indirectly affected by polymerization/depolymerization, structural change, and stretching of the actin filament. Therefore, ND-Lifeact, which realizes the fluorescence observation of the actin filament over long periods, is a novel and feasible analytical material for mechanobiology. Moreover, NVC is known as a probe that quantitatively measures various physical parameters such as pressure, temperature, angle, electric field, and magnetic field using ODMR measurement. Especially, ODMR measurements of magnetic field can be used for high-contrast superresolution imaging, which potentially provide information about nanometric structural changes of actin filament using ND-Lifeact. Hence, we expect to more precisely realize the quantitative measurement of biological phenomena by combining such measurements. The measurement of the ODMR signal shown in this paper resulted not only in selective imaging but also in establishing new avenues for the measurement of various physical parameters accompanied with mechanical stimulation.

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Supporting Information

Figures S1 and S2 are shown in Supporting Information. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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