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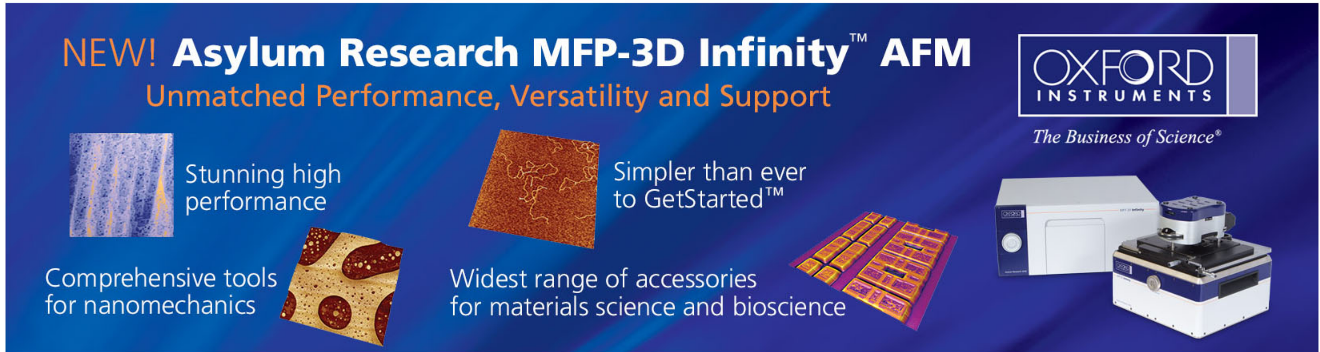

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Direct and *in vitro* observation of growth hormone receptor molecules in A549 human lung epithelial cells by nanodiamond labeling

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This letter presents direct observation of growth hormone receptor in one single cancer cell using nanodiamond-growth hormone complex as a specific probe. The interaction of surface growth hormone receptor of A549 human lung epithelial cells with growth hormone was observed using nanodiamond's unique spectroscopic signal via confocal Raman mapping. The growth hormone molecules were covalent conjugated to 100 nm diameter carboxylated nanodiamonds, which can be recognized specifically by the growth hormone receptors of A549 cell. The Raman spectroscopic signal of diamond provides direct and *in vitro* observation of growth hormone receptors in physiology condition in a single cell level. © *2007 American Institute of Physics*.
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The gene of growth hormone (GH) is expressed not only in the pituitary gland but also in benign and malignant tumors.[1] Meanwhile, the GH receptors have been detected in a variety of cancer cells.[2] It is thought that the GH signal transduction is initiated by the GH-induced growth hormone receptor (GHR) dimerization.[3] Previous studies indicated that both the production of GH from endocrine and autocrine stimulated cancer development via GH-GHR signal transduction pathway.[4] This implies that GH/GHR may be involved in the pathogenesis of human colorectal cancer.[5] Namely, the expression level of GHR may represent the cancer development stage of certain tumors. Therefore, it is important to identify the GHR level in the tumor cells. The detection of growth hormone receptor can be important and the detection technique may facilitate us to identify early phase carcinoma. In this study, we synthesized a highly sensitive nanodiamond-GH complex, which can be used to probe the number of GHR in the single molecular level. This may help us to monitor the status of cancer development in the cellular level.

Recent development in spectroscopic techniques allows high sensitive image detection both *in vitro* and *in vivo* in the cell level. Among various techniques, Raman spectroscopy is recently effectively used in many bio- and medical studies.[6] With confocal configuration, it can achieve high spectral (~ 1 cm^{-1}) and spatial (roughly half of the excitation wavelength) resolution, meaning spectral overlapping can be largely avoided. The advantages of Raman spectroscopy permit the developing of biolabeling techniques for bioapplications using biocompatible nanoparticles with intense and simple Raman signal. To these requests, nanodiamonds respond satisfactorily for its simple Raman signal; they are biocompatible,[7] chemically stable, and convenient for bioconjugation either chemically (covalently or noncovalently)[8,9] or physically (adsorption).[9,10] Typical Raman spectrum of diamond exhibits a sharp peak at 1332 cm^{-1} for phonon mode of the sp^3 bonding carbons. The diamond peak is isolated, and the Raman absorption cross section is large.[11] Therefore this peak can be used as an indicator for the location of diamond.

To demonstrate this, we studied the interaction of carboxylated nanodiamond-GH complex with A549 human lung epithelial cells. Fish growth hormone (recombinant fish growth hormone of the yellow grouper, rEaGH) is covalently bonded to the carboxylated nanodiamond (cND), named as cND-rEaGH. The dissociation constants K_d (the inverse of binding constant) between human GH/hGHR dimmers[12] are 16 and 0.25 nM. In heterogeneous (nonhomologous) GH/GHR binding could be lower by 5- to 500-folds.[13] The K_d between rEaGH/hGHR is about 8 μM in the worst case. In this case the binding constant is still relatively high. Meanwhile, qualitative cell proliferation assay has indicated that the rEaGH interacts with human GHR. The functions of fish growth hormone are similar to those of mammalian growth hormone *in vitro*.[14] To start the preparation, nanodiamond (average diameter 100 nm, GE, USA) was carboxylated via standard procedure of strong acid treatment to create surface functional groups.[15] The rEaGH, the analog of human GH, was synthesized following the methods developed by us previously.[14,16] The synthesized rEaGH was conjugated to cND surface using the reagents of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride and *N*-hydroxysuccinimide (Pierce Led.) and these reagents linked carboxyl group of nanodiamond and amino group of rEaGH and formed a covalent amide bond.[17] The prepared sample was centrifuged at high speed ($\sim$12 000 rpm); the nonconjugated GH in the supernatant was discarded. The sediments were collected and washed several times with de-

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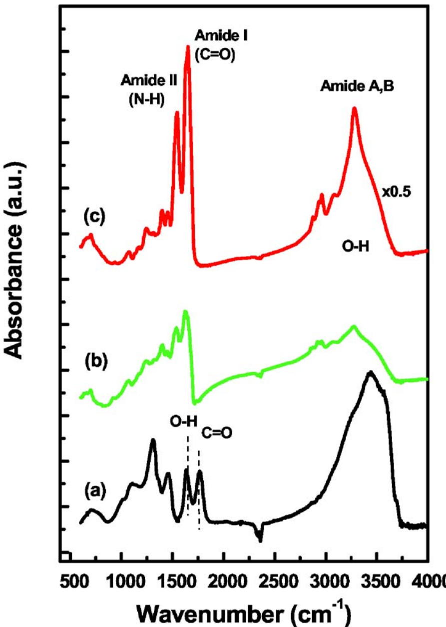

FIG. 1. (Color online) Infrared spectra of (a) cND, (b) rEaGH, and (c) 100 cND-rEaGH.

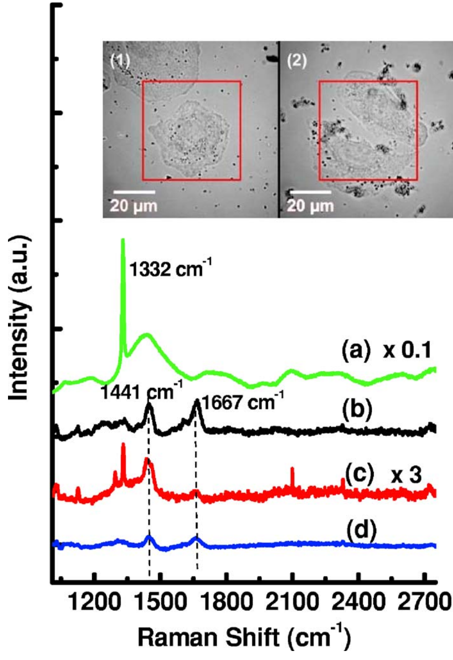

FIG. 2. (Color online) Raman spectra of (a) 100 cND, (b) rEaGH, (c) 100 cND-rEaGH, and (d) A549. Inset: (1) Optical image (×60) of an A549 cell incubated with cND and (2) optical images of two A549 cells incubated with cND-rEaGH.

ionized water to wash away nonconjugated GH. This process was repeated several times to ensure we have cND-rEaGH complex, but not the mixture of cND and rEaGH. The cND-rEaGH complexes were fixed on silicon wafer surface and analyzed with Fourier transform infrared (Bomem 154B, Canada) and confocal Raman spec-troscopy (Witec Alpha 300, Germany, using a 488 nm wavelength laser excitation). The IR measurements assure that proper functional groups are created on the nanodiamond surface. In Fig. 1(a), the typical IR spectrum of cND is presented. The O–H bending near 1630 cm^{-1} and C=O stretching at 1756 cm^{-1} are characteristic for the carboxylic group connected on the nanodiamond surfaces. We have shown that C=O stretching frequency is particle size dependent and sensitive to the local environment.[15] Figure 1(b) was obtained from fish growth hormone (rEaGH); characteristic protein amide peaks are observed: amide I, C=O stretching 1640 cm^{-1}; amide II, primarily N–H bending near 1545 cm^{-1} can be seen.[18] When the rEaGH was conjugated with cND to form cND-rEaGH complex covalently, shown in Fig. 1(c), the C=O disappears and replaces with amide peaks from rEaGH. The IR measurement also ensures that examined sample (cND-rEaGH) did not consist of mixture of rEaGH and cND, but the designed complex cND-rEaGH.

The interaction of A549 cells with cND-rEaGH complex, or with cND, was observed via confocal Raman mapping. Some isolated Raman signals of the cND, rEaGH, and A549 are selected for the mapping of the GH and GHR interaction. Shown in Fig. 2, the Raman shifts near 1440–1460 and 1660–1670 cm^{-1}, correspondingly, are for both the GH from the CH_2 groups and α-helix structure of amide I,[18] and the A549 cell primarily from the CH of lipids and amide I.[19] Figure 2(a) depicts an intense and sharp 1332 cm^{-1} Raman shift for the bulk diamond from the cND. A broad shoulder at 1437 cm^{-1} is from the graphitic structure (sp^2 carbons) existed on the cND surface. Due to large Raman cross section of the sp^2 carbon, the broad peak is visible even only a small fraction of the graphitic structure exists. Figure 2(b) is the Raman spectrum of the GH. Figure 2(c) displays the spectrum for the combination of the rEaGH and cND where both diamond and amide signals exist. Figure 2(d) is the Raman spectrum from a single A549 cell. Both A549 and GH exhibit the same peaks near 1441 and 1667 cm^{-1}. The A549 lung cancer cell line was derived from the lung adenocarcinoma of a 58-year-old Caucasian male. The cells were cultured on Si substrate kept in Petry dish with RPMI-1640 cell growth medium (Invitrogen Co., Carlsbad, CA). The cND-rEaGH (or cND) was then added in necessary quantity to create the cND-rEaGH (or cND only) concentration ~10 μg/ml. The sample was incubated at 37 °C and 5% CO_2 in a humidified incubator for 4 h. The substrate with adhered cells was twice washed with phosphate-buffered saline (pH 7.4) to remove the cND-rEaGH not reacted with GHR or cND not penetrated into cells. Experimentally this time is enough for penetration of cND into cells as well as for cND-rEaGH interaction with cells. We have proven that the GHR proteins were expressed in A549 cells (data not shown). The optical images of the cells are shown in the inset of Fig. 2. In the inset (1), a single cell with cND applied and incubated; in inset (2) the cells were applied with cND-rEaGH and incubated. Both optical images do not reveal any difference. However, Raman spectra reveal that the diamond Raman signal can be easily observed in biosystem in ambient condition without further sample preparation required. In addition, the conjugation of biomolecule does not alter the diamond signal nor does it change the amide signals from the proteins in the biomolecules. When the cNDs were applied to incubate with the A549 cells, they are observed penetrating inside the cell. The exact mechanism of the cND cell endocytosis may be similar to the uptake and pathway mechanism of carbon nanotube.[16] This endocytosis can be observed with Raman mapping. The mapping was performed in an xy plane of 50 $\times 50$ μm^2 area, 1 μm step, and at various z-axis positions. The series of z-axis scans were performed from z $=10$ μm to $z=-10$ μm, 1 μm step, with the $z=0$ arbitrarily

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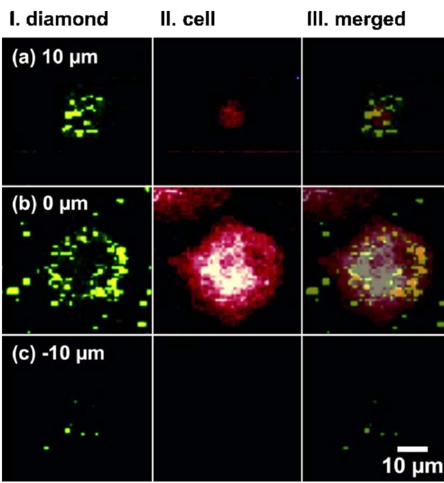

FIG. 3. (Color online) Confocal Raman mapping image of A549 cell and 100 cND. The images shown are at different z position scans: (a) at $z = 10$ μm with diamond collected in 1320–1340 cm^{-1} and cell collected in 1432–1472 cm^{-1}, (b) at $z=0$ μm position, and (c) at $z=-10$ μm position.

set at the Si substrate surface. Figures 3 and 4 illustrate this observation. The optical images of the cell(s) are shown in the insets of Fig. 2. Figure 3 shows only three positions for $z=10$, 0, and -10 μm, respectively (full mapping of all z positions are available but not shown). The images are constructed from a selected spectral line intensity on the Raman spectra that is unique for the samples (ND, GH, GHR, or cell). Therefore, in Figs. 3 and 4 the intensity of each point comes from the selected Raman spectrum intensity collected at this point. Column 1 of Fig. 3 maps the Raman signal of cND, while column 2 is the Raman signal locked on the A549 (the amide peak at 1432–1472 cm^{-1}). As seen in Fig. 3, when the two columns were merged (column 3), the cND signals overlap with the cell, indicating that the cND can penetrate inside the cell. The z position mapping reveals that the cND resides near the nucleus of the cell. However, when the cND-rEaGH complexes were applied the effects are different, as shown in Fig. 4. With the GH conjugated on the

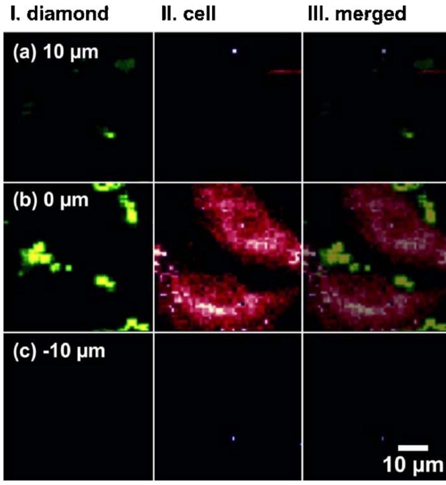

FIG. 4. (Color online) Confocal Raman mapping image of A549 cell and 100 cND-rEaGH carboxylated nanodiamond complexes. The images shown are at different z position scans: (a) at $z=10$ μm with diamond collected in 1320–1340 cm^{-1} and cell collected in 1432–1472 cm^{-1}, (b) at $z=0$ μm position, and (c) at $z=-10$ μm position.

cND, the whole complex resides only on the surfaces of the cell. Hormone-binding domain of growth hormone receptor is extracellular domain.[20] Therefore, the GH/GHR complex forming is on the extracellular part of membrane. This is consistent with our observation. This observation provides strong evidence that growth hormone receptors exist on the surface, and the GH/GHR interaction can be labeled with the diamond Raman signal. This investigation suggests that Raman mapping is a useful technique to observe biomolecule interaction with cells. When this method combines with surface enhanced Raman spectroscopy or surface enhanced resonant Raman spectroscopy, signal can be enhanced by orders of magnitude; it is possible to observe one single molecule interacts with a single cell.[21,22]

In conclusion, this result demonstrates Raman spectroscopic technique has high spectral and spatial resolution in medical applications as well as high sensitivity in detecting molecular interaction in the single cell level. Carboxylated nanodiamond can be a useful platform to conjugate biomolecules for use as nanobioprobe. The growth hormone receptor molecules in A549 human lung epithelial cells were observed by nanodiamond labeling.

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