Non-destructive electron microscopy imaging and analysis of biological samples with graphene coating

Jong Bo Park$^{1,9}$, Yong-Jin Kim$^{1,2,9}$, Seong-Min Kim$^3$, Je Min Yoo$^1$, Youngsoo Kim$^{4,9}$, Roman Gorbachev$^2$, I I Barbolina$^{1,9}$, Sang Jin Kim$^1$, Sangmin Kang$^4$, Myung-Han Yoon$^3$, Sung-Pyo Cho$^1$, Konstantin S Novoselov$^1$ and Byung Hee Hong$^{1,9}$

$^1$ Department of Chemistry, College of Natural Science, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul, 151–747, Korea
$^2$ Department of Physics and Astronomy, University of Manchester, Manchester, M13 9PL, UK
$^3$ Department of Materials Science and Engineering, Gwangju Institute of Science and Technology, Gwangju, 500–712, Korea
$^4$ Department of Physics and Astronomy, College of Natural Science, Seoul National University, Seoul, 151–747, Korea
$^5$ Faculty of Life Sciences, Michael Smith Building, Oxford Road, Manchester, M13 9PT, UK
$^6$ Technical Service Consultants Ltd, Microbiology House, Fir Street, Heywood, OL10 1NW, Lancashire, UK
$^7$ National Center for Inter–University Research Facilities, Seoul National University, Seoul, 151–742, Korea
$^8$ Graduate School of Convergence Science and Technology, Seoul National University, Suwon, 443–270, Korea

Abstract

In electron microscopy (EM), charging of non-conductive biological samples by focused electron beams hinders their high-resolution imaging. Gold or platinum coatings have been commonly used to prevent such sample charging, but it disables further quantitative and qualitative chemical analyses such as energy dispersive spectroscopy (EDS). Here we report that graphene-coating on biological samples enables non-destructive high-resolution imaging by EM as well as chemical analysis by EDS, utilizing graphene’s transparency to electron beams, high conductivity, outstanding mechanical strength and flexibility. We believe that the graphene-coated imaging and analysis would provide us a new opportunity to explore various biological phenomena unseen before due to the limitation in sample preparation and image resolution, which will broaden our understanding on the life mechanism of various living organisms.

Comprehensive understanding of biological objects—their chemical, physiochemical and biological characteristics—can be effectively achieved through electron microscopy (EM) analysis [1–4], preferably without any fixation or auxiliary surface treatment. Scanning electron microscope (SEM) and transmission electron microscope (TEM) are increasingly more employed as they provide direct imaging of specimen’s morphological structures with high-resolution [5, 6]. Recently, EM analysis has extended its use to construct three-dimensional structure of the biological specimen with combination of serial block-face sectioning or focused ion beam [7, 8]. In addition, the unique interaction between electron beams and specimen enables various physical and chemical analyses such as energy dispersive spectroscopy (EDS), electron probe micro analysis, and electron energy loss spectroscopy [9, 10]. Nevertheless, charge accumulation by electron beams and shrinkage of samples by dehydration in vacuum have always hindered EM-mediated biological studies as it distorts the morphological and chemical characteristics of the specimens [11–16]. For these reasons, various coating and sample preparation methods for EM analysis have been developed to enhance the image contrast from non-conducting biological specimen [1, 5, 17–28]. In particular, a metal- and carbon-coating methods have been widely employed to dissipate the accumulated charges on non-conducting surface [23–27].

However, the relatively thick coating layer hampers from studying the fine structures of the specimen at nanometer scale because of the large size of metal or
carbon grains. In addition, x-ray fluorescence signals required for EDS analysis are screened by metal layers [29]. Furthermore, it is usually difficult to use the metal-coated samples for further analyses such as TEM that requires electron-transparency. Here we report that graphene-coating on biological samples enables non-destructive high-resolution imaging by SEM and TEM as well as chemical analysis by EDS, utilizing graphene’s transparency to electron beams, high conductivity, outstanding mechanical strength and flexibility [30–32].

Recent progresses in large scale synthesis of high quality graphene films using chemical vapor deposition (CVD) methods have widened its potential in practical device applications as well as unique interests in basic scientific researches [33–36]. The feasibility of the large scale fabrication of continuous graphene films as well as easy transfer onto diverse biological objects opens up a unique opportunity to create new hetero-interfaces or interfaces with non-conducing biological samples. As demonstrated in a recent work [37], the in situ high-resolution EM imaging of nanocrystal growth has been achieved by using graphene liquid cells to encapsulate nanoscale materials as well as their environment (i.e. liquid) and separate them from the vacuum environment. In this regard, graphene mediated coating on biological samples can provide high-resolution EM imaging and chemical analysis due to the excellent electron and heat flow thorough the graphene and electron-transparency [44]. Here, with taking all these advantages of graphene films, we have employed continuous graphene films as coating for biological samples and exploited them for non-destructive high-resolution EM imaging and chemical analysis.

As schematically displayed in figure 1, the unique feasibility and availability of continuous graphene films at large scale enables the conformal coating of biological objects including leaves, ants, spiders, neuron cells, Escherichia coli (E. coli), proteins, and polypeptides whose sizes range from several centimeters down to few nanometers. Atomically-thin and electrically-conducting graphene membranes were prepared on non-conducting biological surface by isolating graphene films from copper (Cu) foils after CVD growth, followed by conformal coating onto biological samples as illustrated in figure 1(c). Compared to other conventional sample preparation methods including fixation and metal sputter coating (figures 1(a) and (b)), the present method based on graphene coating is relatively simple, bio-friendly and non-destructive, which is particularly advantageous for preserving the chemical information of samples for further experiments.

Monolayer graphene film was synthesized on high-purity Cu foil using CVD method (please see supplementary materials). Continuous graphene films coated with a poly(methyl methacrylate) (PMMA) layer can be isolated from Cu foils and transferred to a target surface after wet chemical etching [35]. The PMMA was removed by using acetone before the Cu etching. The number of graphene layers was controlled by repeating this transfer process. We found that triple-layered (3-layer) graphene films provide optimum electron transparency and mechanical stability for SEM analysis (figures S1–S3). The biological specimens were cleaned and positioned onto a metallic sample stage for SEM imaging. The 3-layer graphene sheets were then transferred on top of the biological specimen by scooping from bottom side, followed by drying in a desiccator.

To demonstrate the advantages of using graphene membrane for SEM imaging, we have selected several representative biological specimens (ants, bee’s wings, water fleas, E. coli and ferritin) that are different in terms of size, surface hardness, and morphology. The 3-layer graphene mostly covered these millimeter to nanometer sized samples, and only shows minor fractures around needle-like structures (figure 2(c)). In contrast, the use of graphene oxide (GO) and reduced GO resulted in incomplete coating due to their poor mechanical strength and difference in hydrophobicity (figure S4). An SEM imaging on a carbon coated samples also showed large carbon grains that distort the sample’s morphology (figure S5). The high-magnification FE-SEM images of a graphene-coated ant clearly show not only unique micro-patterns but also nanopores as small as 40 nm (figure 2(b)) that are invisible in platinum (Pt)-coated or carbon-coated samples (figures 2(i) and S5). Such fine and clear observation of the surface structures implies that the adhesion between graphene and the sample (mostly by van der Waals interaction) is strong enough to maintain its morphology [38] and stable up to acceleration voltage of 20 kV (figure S6). The needle-like structures on bee’s wings result in punctures on graphene, but the surface still shows conformal graphene coating that enables stable SEM imaging (figure 2(c)). We also performed SEM imaging on a 1.5 mm long water flea (Daphnia pulex) covered with 3-layer graphene films. High-magnification SEM images of the water flea (area P1 in figure 2(d)) clearly display the unique features of a water flea on its dorsal carapace (figure 2(e)). Interestingly, the graphene film mostly covers the needle-like surface on its antenna without much tearing (figure 2(f)).

The advantages of graphene coating compared with a conventional metal coating method were demonstrated under identical conditions (figures 2(g)–(i)). We observed that the bare gaster surface of an ant is strongly charged even at low acceleration voltages (<2 kV) (figure 2(g)), and the bare eye surface is immediately burning at 5 kV, while the graphene-coated area does not show any damage even with high acceleration voltages up to 20 kV (figures 2(g), (h), and S6).

Unlike the above mentioned hard-surfaced insects, soft biological objects such as tissues, cells and
bacteria need an additional treatment for EM analysis, including aldehyde fixation, osmium tetroxide staining, and critical point drying. In this process, the use of organic solvents often distorts the samples’ original contents and disables further qualitative or quantitative chemical analyses. In this regard, the simple graphene-coating method can be advantageous because biological samples close to their native structures can be imaged and preserved for further analyses. If combined with conventional fixation methods, it would be more effective for the high-resolution EM imaging of biological samples (figure S7). We also demonstrate that common bacteria, *E. coli*, cultured in a liquid medium can be imaged after monolayer graphene-coating that protects *E. coli* from sudden vacuum drying as well as e-beam damage (figures 2(i)–(l)) in SEM. Another graphene layer on bottom side was used to seal the liquid environment by $\pi - \pi$ interaction with top graphene layer. Recently, an environmental SEM (ESEM) has been utilized to observe the native structures of biological samples without conductive coating, but its resolution is still limited due to the charging problem associated with low conductivity of biological surfaces (figures S7 and S8)\(^{[39-41]}\). We also observed that untreated *E. coli* shrank by dehydration in a vacuum chamber during ESEM imaging (figure S9). On the other hand, the graphene coating not only provides higher resolution than ESEM but also stabilizes the liquid-containing samples that can be easily damaged by intense electron beams.

We also performed EM imaging of ferritin, an intracellular protein that stores and releases iron to control the iron concentration in living organisms\(^{[42]}\). The ferritin particles in water are encapsulated between two monolayer graphene films. The individual ferritin particles are clearly observed in SEM, in which the iron cores look brighter (figure 2(m)). In a low-magnification TEM image, spherical protein shell as well as hydrous ferric oxide cores were identified from their different contrast, and at high-magnification, the lattice fringe of the iron core was clearly resolved with atomic resolution in an aqueous medium (figure 2(o)). Likewise, we also demonstrate that the hydrated structure of plasmid deoxyribonucleic acids of *E. coli* sandwiched between graphene layers can be successfully imaged by SEM and TEM (figure S10).

We compared the performances of graphene-coating and Pt-coating methods in chemical analysis by EDS. All the experimental conditions and parameters including spot sizes and signal collection time were identical. The results showed that EDS signals from graphene-coated samples (figure 3(a)) are 2–3 times stronger than Pt-coated samples (figure 3(c)), which facilitates the qualitative and quantitative chemical analyses on nitrogen-containing chitin (from ants) and oxygen-rich cellulose (from leaves). The non-destructive analysis enabled by the graphene coating is particularly efficient for element-specific EDS mapping. The water flea sample was fed with 25 nm cerium oxide nanoparticles (CeO\(_2\) NPs) to stain its digestive pathway (please see supplementary materials for experimental details). The CeO\(_2\) NPs are clearly visualized in the Ce-selective EDS mapping of

---

**Figure 1.** Schematic illustration of various biological objects in different scales and coating methods for EM analysis. (a) and (b), Conventional coating methods of non-conducting biological samples. Soft biological samples such as cells and bacteria require complicated coating processes including aldehyde fixation, osmium tetroxide fixation, dehydration, critical point drying, staining, metal coating, etc. Hard-surfaced biological samples such as insects and plants are usually coated with Au, Pt by vacuum sputtering. The metal coating is simple, but it disables high-resolution imaging and analysis. (c) Simple coating process using graphene floating on water surface. The ambient drying process allows the conformal coating of graphene on sample surface.
the graphene-coated water flea, while the Pt-coated sample does not show clear EDS signals (figures 3(d) and (e)). The other EDS analyses also indicate that the graphene-coated method is superior to Pt-coating in terms of signal intensity (figure S11). We attribute the signal reduction in the Pt-coated samples to the absorption and scattering of incident electrons and x-ray fluorescence radiation by thick Pt layers, which will be further discussed in figure 4.

The outstanding performance of atomically thin graphene membrane as protective coating for EM analysis was theoretically confirmed by Monte Carlo simulations (please see supplementary materials for detailed methods). The 1 nm graphene-coated chitin (Gr/chitin) was compared with 10 nm Pt coated chitin (Pt/chitin). As seen in the electron trajectory images (figure 4(a)), incident electrons can easily pass through the thin graphene and penetrate deep into the chitin.

Figure 2. SEM images of various biological samples covered with graphene films. (a) and (b) Low- and high-magnified SEM images of a graphene-coated ant. (c) SEM images of graphene-coated bee’s wing, where about 30 μm sized needle-like structures are uniformly arrayed. (d)–(f) Low- and high-magnified SEM images of a graphene-coated water flea, respectively. The graphene film exhibits conformal contact with the non-flat surfaces of biological samples. Acceleration voltages for A to F, 2 kV. (g)–(i) Representative SEM images showing the comparison between bare, graphene-coated, and Pt-coated samples. The graphene coating enables the stable SEM imaging of sub-10 nm features on the surface, while the Pt-coated sample shows distorted morphology covered with Pt nanoparticles. (j) Optical microscopic image of graphene-coated E. coli. (k) and (l) Low- and high-magnification SEM images corresponding to (j) obtained with acceleration voltage at 2 kV. (m) SEM image of graphene-coated ferritin proteins at 2 kV. (n) and (o) Low- and high-magnified TEM images of ferritin with graphene coating at 200 kV, respectively.
layer, while the Pt layer blocks electron penetration because of the large nucleus radius (i.e. large electron scattering cross-section) of Pt \((Z = 78)\) (see supplementary figure S12 for cross-section calculations). As the acceleration voltage increases from 2 to 10 kV, the maximum penetration depth of electrons increased from 140 to 2250 nm for Gr/chitin. This indicates that graphene membrane is more transparent to lower accelerating voltages. On the contrary, the electrons irradiated to Pt/chitin show less penetration depths with larger scattering angles. The amount of penetrated electrons is directly related to the x-ray signals.
(figure 4(c)), resulting in large difference in x-ray absorption intensity between Gr/chitin and Pt/chitin. Figure 4(b) shows the cross-section profiles of energy loss along the simulated electron trajectories [43], which is related to the intensity of EDS signals. From 2 and 5 kV, most of the energy loss happens inside the 10 nm Pt layer. Major energy loss still occurs near the Pt layer at 10 kV. On the other hand, energy loss in Gr/chitin mostly takes place inside the chitin layer even at 2 kV, indicating that the graphene is almost free from electron energy loss and background EDS signals. Figure 3(e) shows the depth profiles of Rho-Z x-ray intensity of carbon for Gr/chitin (red) and Pt/chitin (gray). As the accelerating voltage increases from 2 to 10 kV, the total x-ray intensities for Pt/chitin and Gr/chitin increased from 6 to 259 and from 87 to 470, respectively (see also supplementary figure S14 for the simulations for nitrogen and oxygen analysis). This indicates that graphene is superior to Pt for protective coating for EDS analysis. The experimental EDS spectra with varying accelerating voltages coincide with the simulation results (figure S15).

In conclusion, we demonstrated that graphene’s outstanding mechanical strength, conductivity, flexibility, and transparency to electron beams enable the simple and non-destructive imaging and analysis of various biological samples with high resolution that can be hardly achieved in bare or metal-coated samples. The graphene coating effectively prevent charge accumulation by spreading e-beam induced charges and heats over large surface area, and the high mechanical strength and flexibility of graphene allows conformal coating by excellent adhesion with various biological interfaces. We believe that the graphene-coated imaging and analysis would provide us a new opportunity to explore various biological phenomena unseen before due to the limitation in sample preparation and image resolution, which will broaden our understanding on the life mechanisms of various living organisms.

Acknowledgments

This research was supported by the Nano Material Technology Development Program (2012M3A7B4049807) through the National Research Foundation of Korea funded by the Ministry of Science, ICT and Future, Korea. TEM samples were analyzed using the transmission electron microscope (JEM-2100, JEOL) installed at the National Center for Inter-University Research Facilities (NCIRF) at Seoul National University. The authors thank to the staff and crew of the National Center for Inter-university Research Facilities at Seoul National University for assistance with TEM experiments. This work was supported by the Brain Korea 21 Plus Project. KSN is grateful to the Royal Society, European Research Council and EC-FET European Graphene Flagship for support.

References

[1] Hayat M A 2000 Principles and Techniques of Electron Microscopy: Biological Applications 4th edn (Cambridge: Cambridge University Press)
[2] Fratzl P 2003 Cellulose and collagen: from fibres to tissues Curr. Opin. Colloid Interface Sci. 8 32–9
[3] Lucic V, Forster F and Baumeister W 2005 Structural studies by electron tomography: from cells to molecules Annu. Rev. 74 833–65
[4] Midgley P A, Ward E P W, Hungria A B and Thomas J M 2007 Nanotomography in the chemical, biological and materials sciences Chem. Soc. Rev. 36 1477–94
[5] Bozola J and Russell L D 1992 Electron Microscopy: Principles and Techniques for Biologists (Boston: Jones and Bartlett Publishers)
[6] Schatten H 2013 Scanning Electron Microscopy for the Life Sciences (Cambridge: Cambridge University Press)
[7] Denk W and Horstmann H 2004 Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure PLoS Biol. 2 1900–9
[8] Bushby A J et al 2011 Imaging three-dimensional tissue architectures by focused ion beam scanning electron microscopy Nat. Protocols 6 545–58
[9] Joy D C, Alton D, Romig J and Goldstein J I 1986 Principles of Analytical Electron Microscopy (New York: Plenum)
[10] Brydson R 2001 Electron Energy Loss Spectroscopy (London: Taylor and Francis)
[11] Royall C P, Thiel B L and Donald A M 2001 Radiation damage of water in environmental scanning electron microscopy J. Microsc. 204 185–95
[12] Henderson R 1992 Image-contrast in high-resolution electron-microscopy of biological macromolecules—Tmn in ice Ultramicroscopy 46 1–18
[13] Parsons D F 1962 An electron microscope study of radiation damage in mouse oocyte J. Cell Biol. 14 31–48
[14] Gibson J B, Goland A N, Milgram M and Vineyard G H 1960 Dynamics of radiation damage Phys. Rev. 120 1229–53
[15] Isaacson M, Johnson D and Crew A V 1973 Electron-beam excitation and damage of biological molecules—it’s implications for specimen damage in electron microscopy Radiat. Res. 55 203–24
[16] Coslett V E 1978 Radiation damage in the high resolution electron microscopy of biological materials: a review J. Microsc. 113 113–29
[17] Williams R C and Wyckoff R W G 1945 Electron shadow micrography of the tobacco mosaic virus protein Science 101 594–6
[18] Bradley D E and Juniper B E 1957 Electron microscopy of leaf surfaces Nature 180 330–1
[19] Peters K R 1977 Stereo surface replicas of culture cells for high-resolution electron-microscopy J. Ultra. Mol. Struct. 61 115–23
[20] Porter K R, Claude A and Fullam E F 1945 A study of tissue culture cells by electron microscopy—methods and preliminary observations J. Exp. Med. 81 233–46
[21] Hall C E, Jakus M A and Schmitt F O 1945 The structure of certain muscle fibrils as revealed by the use of electron stains J. Appl. Phys. 16 659–65
[22] Hall C E 1955 Electron densitometry of stained virus particles J. Biophys. Biochem. Cytol. 1 1–12
[23] Echlin P and Hyde P J W 1975 The rationale and mode of application of thin films to non-conducting materials Scan. Electron Microsc, 1 137–46
[24] Peters K R 1979 Scanning electron microscopy at macromolecular resolution in low energy mode on biological specimens coated with ultra thin metal films Scan. Electron Microsc. 2 133–48
[25] Peters K R 1982 Conditions required for high-quality high magnification images in secondary electron-l scanning electron-microscopy Scan. Electron Microsc. 4 1359–72
[26] Hermann R and Muller M 1991 High-resolution biological scanning electron microscopy—a comparative study of low-temperature metal-coating techniques J. Electron Microsc. Tech. 18 440–9
[27] Stokroos I, Kalicharan D, Van der Want J J L and Jongebloed W L 1998 A comparative study of thin coatings of Au/Pd, Pt and Cr produced by magnetron sputtering for FE-SEM J. Microsc. 189 79–89
[28] Peters K R 1986 Metal Deposition by High-Energy Sputtering for High Magnification Electron Microscopy (Berlin: Springer)
[29] Reimer L 1998 Scanning Electron Microscopy: Physics of Image Formation and Microanalysis (Berlin: Springer)
[30] Geim A K and Novoselov K S 2007 The rise of graphene Nat. Mater. 6 183–91
[31] Meyer J C et al 2007 The structure of suspended graphene sheets Nature 446 60–3
[32] Lee C, Wei X D, Kysar J W and Hone J 2008 Measurement of the elastic properties and intrinsic strength of monolayer graphene Science 321 385–8
[33] Kim K S et al 2009 Large-scale pattern growth of graphene films for stretchable transparent electrodes Nature 457 706–10
[34] Li X S et al 2009 Large-area synthesis of high-quality and uniform graphene films on copper foils Science 324 1312–4
[35] Bae S et al 2010 Roll-to-roll production of 30-inch graphene films for transparent electrodes Nat. Nanotechnol. 5 574–8
[36] Bae S, Kim S J, Shin D, Ahn J H and Hong B H 2012 Towards industrial applications of graphene electrodes Phys. Scr. T146 014024
[37] Yuk J M et al 2012 High-resolution EM of colloidal nanocrystal growth using graphene liquid cells Science 336 61–4
[38] Radic S et al 2013 Competitive binding of natural amphiphiles with graphene derivatives Sci. Rep. 3 2273
[39] Donald A M 2003 The use of environmental scanning electron microscopy for imaging wet and insulating materials Nat. Mater. 2 511–6
[40] Muscariello L et al 2005 A critical overview of ESEM applications in the biological field J. Cell. Physiol. 205 328–34
[41] Kirk S, Skepper J and Donald A M 2009 Application of environmental scanning electron microscopy to determine biological surface structure J. Microsc. 233 205–24
[42] Theil E C 1987 Ferritin—structure, gene-regulation, and cellular function in animals, plants, and microorganisms Annu. Rev. Biochem 56 289–315
[43] Drouin D et al 2007 CASINO V2.42—a fast and easy-to-use modeling tool for scanning electron microscopy and microanalysis users Scanning 29 92–101
[44] Jeon J, Lodge M S, Dawson B D, Ishigami M, Shewmaker F and Chen B 2013 Superb resolution and contrast of transmission electron microscopy images of unstained biological samples on graphene-coated grids Biochim. Biophys. Acta 1830 3807–15