METHOD

Histone chaperone-mediated co-expression assembly of tetrasomes and nucleosomes

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The nucleosome, a basic unit of chromatin found in all eukaryotes, is thought to be assembled through the orchestrated activity of several histone chaperones and chromatin assembly factors in a stepwise manner, proceeding from tetrasome assembly, to H2A/H2B deposition, and finally to formation of the mature nucleosome. In this study, we demonstrate chaperone-mediated assembly of both tetrasomes and nucleosomes on the well-defined Widom 601 positioning sequence using a co-expression/reconstitution wheat germ cell-free system. The purified tetrasomes and nucleosomes were positioned around the center of a given sequence. The heights and diameters were measured by atomic force microscopy. Together with the reported unmodified native histones produced by the wheat germ cell-free platform, our method is expected to be useful for downstream applications in the field of chromatin research.

In eukaryotic nuclei, genomic DNA is organized into chromatin. The basic building block of chromatin is the nucleosome, containing about 147 bp double-stranded DNA wrapping around a histone octamer composed of two copies each of H2A, H2B, H3, and H4 in a left-handed superhelix [1–3]. The open and closed chromatin higher order structures are dictated by local and genome-wide assembly and disassembly of the nucleosome, which in turn regulate DNA replication, repair, gene expression, and others [4–6]. In the nuclei, the nucleosome is formed in a sequential process mediated by histone chaperones and chromatin assembly factors [7]. Two copies of the H3/H4 dimer are recruited to form an (H3/H4)\textsubscript{2}-DNA complex (tetrasome), followed by deposition of H2A/H2B dimers [8]. The mechanisms and kinetics of nucleosome formation have been described, and the roles of several chromatin assembly factors have been studied in detail [9,10]. Furthermore, it has been indicated that disassembly of H2A/H2B is actively proceeded during chromatin maintenance in the cell nucleus [11]. For instance, eviction of H2A/H2B from nucleosome could release the torsions created by the adjacent chromatin structure. One of the histone chaperones, nucleosome assembly protein 1 (NAP1), is known for its function in aiding nucleosome assembly at physiological ionic strength and is highly conserved among many species of eukaryotes [12–17]. \textit{In vitro} studies described the thermodynamics of NAP1-mediated nucleosome assembly, and one of the important roles of NAP1 besides forming tetrasomes is to prevent non-nucleosomal interaction of H2A/H2B dimers during nucleosome formation [9,18]. These studies and other chromatin function-related research essentially utilize

Abbreviations
AFM, atomic force microscopy; MNase, Micrococcal nuclease; NAP1, nucleosome assembly protein 1.
recombinantly expressed, refolded, and preassembled core histones; thus, functions of chromatin-associated proteins such as NAP1 during nucleosome formation under physiological conditions needs to be further tested.

Previously, we reported a novel in vitro chromatin reconstitution method using a wheat germ cell-free co-expression system, in which mRNAs encoding four core histones were co-expressed with histone chaperones and ATP utilizing factors in the presence of a template circular DNA [19]. This method enables simultaneous histone synthesis and chromatin assembly under near physiological conditions in vitro. Additionally, the recent report from our group showed that the synthesized histones by a wheat germ cell-free synthesis lacked any detectable posttranslational modification [20].

In this study, we sought to make our method more applicable for downstream applications to study chromatin assembly and disassembly dynamics under near physiological conditions. To this end, homogeneous Drosophila melanogaster tetrasomes and nucleosomes were assembled on the well-defined Widom 601 nucleosome positioning sequence in a histone chaperone-mediated manner [19,21]. Furthermore, the affinity-purified reconstituted tetrasomes and nucleosomes were analyzed by restriction enzyme digestion and micrococcal nuclease digestion, respectively. The typical heights and diameters of purified tetrasomes and nucleosomes were determined by atomic force microscopy. Overall, the current study provides evidence that our histone chaperone-mediated co-expression assembly of tetrasome and nucleosome enables preparation of a positioned homogeneous complex on the Widom 601 sequence, useful for downstream applications.

Materials and methods

Cloning

The DNA fragments of 185 bp and 307 bp containing the Widom 601 sequence at the center, 601_185 bp, and 601_307 bp, were amplified from pGEM-3z/601 (Addgene, MA, USA) by PCR with the following primer pairs 5'-GTCGCTGTTCAATCATGCG-3' and 5'-GACCTTATACCGCACC-3' for 601_185bp, and 5'-TCGCTTACCCGGGATATCC-3' and 5'-CAGGATCTGAAATGGG-3' for 601_307bp. Both fragments were purified by phenol-chloroform-IAA (25 : 24 : 1, v/v) extraction followed by ethanol precipitation and resuspended in 5 mM Tris/HCl buffer. The plasmid constructs harboring Drosophila melanogaster core histones and NAP1 were prepared as described previously [19]. In order to purify the reconstituted core histones-DNA complexes, the histone H4 was designed to possess the N-terminal His6-tag, followed by a tobacco etch virus protease cleavage site [22]. The His6-tag coding H4 gene was cloned into a pEU expression vector by a polymerase incomplete primer extension (PIPE) [23] using the following primers, 5'-GAACTTGTACCTC-CAATCTGGACGTGGTAAAGGCC-3' and 5'-TTTTATC-CATTCTAATAGTTAACCCCGCAAGCC-3', and pEU_DmH4 [19] was used as a template for the histone H4 coding gene. The PIPE vector fragment was prepared by PCR reaction using the following primers, 5'-CTTTGAAAGTTG-GATAAC-3' and 5'-TTGGAAGTACAGGTTC-3'. Both PCR products were mixed and immediately transformed into JM109 competent cells (Takara Bio, Shiga, Japan), and transformants were sequence verified (Eurofins Genomics, Tokyo, Japan) by the same primer used to amplify the insert DNA.

In vitro transcription

The plasmid harboring each histone or NAP1 was in vitro transcribed individually, as described previously [19]. All reagents were purchased (CellFree Sciences, Yokohama, Japan). Briefly, 2 μg plasmid DNA was mixed with 20 U SP6 RNA polymerase, 20 U RNase inhibitor, 2.5 mM NTP mix, and 5x transcription buffer in a total reaction volume of 20 μL, and incubated for 4 h at 37 °C. The concentration of synthesized mRNA was quantified by UV spectrophotometry (DS11, DeNovix, Wilmington, DE, USA) after DNase I treatment for 30 min at 37 °C. Transcribed mRNAs were purified as previously described [19] and resuspended in RNase-free water (Nippon Gene, Tokyo, Japan). Histone mRNAs were premixed before translation with a 1:1 ratio of H3:H4 for tetrasome assembly and a 2.5 : 2.5 : 1 : 1 ratio of H2A : H2B : H3 : H4 for nucleosome assembly.

In vitro tetrasome and nucleosome assembly reactions

The tetrasome assembly reaction was performed by mixing 5 μL wheat germ cell-free extract (WEPRO7240H Lot: 20CR02MN, CellFree Sciences), 4.4 μg H3/H4 mRNA mixture, 8.8 μg NAP1 mRNA, 0.4 μg creatine kinase, and 0.25 μg 601_185bp in a total of 10.7 μL reaction mixture. To form a diffusible bilayer reaction [24], the reaction mixture was carefully applied under the top layer consisting of 103 μL 1 × SUB-AMIX. A co-expression chromatin assembly reaction was performed for 6 h at 26 °C. For the nucleosome assembly reaction, 17.6 μg of the premixed four core histone mRNAs was used in the presence of either 0.25 μg 601_185 bp or 0.25 μg 601_307 bp sequence. The composition of reaction and protocol are otherwise the same as the tetrasome assembly reaction, described above. For AFM measurement, the nucleosome assembled on a 601_307 bp DNA fragment was used. Assembled tetrasomes and nucleosomes were incubated for 1 h at 55 °C to eliminate non-specific histones-DNA interactions [25].
Affinity purification of assembled tetrasome and nucleosome

The assembled tetrasomes and nucleosomes were purified using a TALON Magnetic Beads (Takara Bio) via the His6-tag designed at the N-terminal of histone H4. Briefly, 50 µL TALON magnetic beads were rinsed with 300 µL deionized water and then equilibrated with 300 µL equilibration buffer (10 mM Tris/HCl pH7.4, 40 mM NaCl), and the supernatant was removed using a magnetic rack. 113.7 µL of the assembled sample was incubated with the beads for 1 h at 5 °C on a rotator. The supernatant was carefully removed, and the beads were washed 2 times with 500 µL wash buffer (10 mM Tris/HCl pH7.4, 40 mM NaCl, 10 mM Imidazole). Purified tetrasomes and nucleosomes were eluted in 25 µL elution buffer (10 mM Tris/HCl pH7.4, 40 mM NaCl, 250 mM Imidazole), in the presence of 500 µL tetrasomal DNA on a polyacrylamide gel by visual inspection.

Restiction enzyme digestion of reconstituted tetrasomes

A 60 U PmlI (New England Biolabs, Beverly, MA, USA), 30 U BsiW1 (New England Biolabs), or 30 U NolI (Takara Bio) was added to the 50 ng 601_185 bp or the purified tetrasome containing ~ 15 ng DNA in an appropriate buffer system. The restriction enzyme digestion using PmlI and NolI was performed for 1 h at 37 °C, and BsiW1 reaction was carried out for 1 h at 55 °C. After the reaction, the sample was deproteinized by phenol–chloroform-IAA extraction and purified by ethanol precipitation, then resuspended in HD buffer (25 mM HEPES, 1 mM DTT, pH7.6) with trace amounts of RNase A and run on a 10% native polyacrylamide gel, followed by ethidium bromide staining. The band intensities were measured by the Gel-Doc system (Bio-Rad, Hercules, CA USA). The following equation calculated the %protection from PmlI and BsiW1 digestions in the presence of tetrasome relative to naked DNA.

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\text{%protection} = \left( \frac{\text{Remaining tetrasomal DNA}}{\text{(Intact DNA)} - \text{(Remaining naked DNA)}} \right) \times 100\%.
\]

Micrococcal nuclease assay

Purified nucleosomes were mixed with 0, 0.08, 0.4, or 1.6 U of Micrococcal nuclease (New England Biolabs) in the presence of 2.5 mM CaCl2. The reaction was incubated for 10 min at 37 °C, then halted by adding 5 mM EDTA, followed by phenol–chloroform-IAA extraction and ethanol precipitation. Samples were analyzed by a 3% TBE agarose gel in a 1xTBE buffer system and visualized by ethidium bromide staining.

Atomic force microscopy

The surface of mica (TED PELLA, INC, Redding, CA, USA) was treated with 1-(3-aminopropyl) silatrane (APS) as described previously [26] to immobilize the purified ~ 0.5 nm tetrasome or nucleosome samples. Samples were placed on the mica for 5 min at room temperature, then rinsed with deionized water, and dried under nitrogen flow. AFM (NanoWizard III, JPK Instruments, Berlin, Germany) was used in tapping mode to scan the samples in the air with a silicon probe (OMCL-AC160TS-C3; Olympus, Tokyo, Japan). The scanning rate was 8.0 Hz over a scan area of 1 µm. The heights and diameters of tetrasomes (n = 100) or nucleosomes (n = 100) were measured using JPKSPM Data Processing software (JPK Instruments). The diameters of tetrasomes and nucleosomes were calculated by subtracting 2 x 7 nm (tip radius) from the observed diameters, accordingly to a manufacturer. Thresholds for tetrasomes and nucleosomes were made based on both height and diameter. For example, we considered the particles as tetrasomes when they meet criteria of both height and diameter (0.6 nm ≤ height ≤ 2.8 nm and 6 nm ≤ diameter ≤ 18 nm). Similarly, the particles satisfied 1.8 nm ≤ height ≤ 3.4 nm and 12 nm ≤ diameter ≤ 24 nm were considered as nucleosomes.

Results

Tetrasome and nucleosome assembly using wheat germ co-expression system

Previously, we reported a method to reconstitute chromatin by co-expressing four core histones and chromatin assembly factors using a wheat germ cell-free system [19]. To improve this technology for more applicable protocols in the area of chromatin research, we aimed to assemble Drosophila mononucleosome and mononucleosome on a well-described Widom 601 sequence followed by affinity purification using N-terminal His6 tag on histone H4 accordingly to manufacturer’s instructions [27]. A flowchart of this study from a preparation of mRNAs, co-expression/histone-DNA complex assembly reactions, followed by purification is shown in Fig. 1. A pair of H3 and H4-coding mRNAs, or a set of four core histone-coding mRNAs is used together with NAP1-coding mRNA, and they were co-expressed in the presence of a short double-stranded DNA possessing the Widom 601 sequences at the center.
Characterization of assembled tetrasomes

First, we tested whether a tetrasome can be assembled by our method. A pair of H3 and H4 mRNAs was co-expressed with NAP1 mRNA in the presence of 601_185 bp for 6 h at 26 °C, followed by affinity purification. In the absence of H3/H4 mRNAs, neither mobility shift nor free DNA was determined by electrophoretic mobility shift assay (EMSA) as was expected (Fig. 2A). In contrast, a tetrasomal DNA was found by EMSA when a complete tetrasome reconstitution and following purification were performed. The amount of reconstituted tetrasome before and after purification was estimated to be 145.4 ± 21.1 ng per reaction (n = 3) and 17.6 ± 0.7 ng per reaction (n = 3), respectively. Thus, ~12% of tetrasome was purified by affinity purification. By considering the migration of tetrasomes on the gel, it was suggested that only one tetrasome is formed on a given sequence. To address the position of the assembled tetrasome on a 601_185 bp, the following restriction enzymes, PmlI, BsiWI, and NotI, were used for the digestion of assembled and purified tetrasomes (Fig. 2B). The cleavage sites of each restriction enzyme are shown in Fig. 2B, so that detected length from the end products should give us an idea of where roughly the tetrasome is positioned on the 601_185 bp. Tetrasomes were digested by each restriction enzyme, and then, end products were analyzed on a 10% native polyacrylamide gel (Fig. 2C and D). The digestion either by PmlI or BsiWI on a tetrasome was significantly reduced (Fig. 2D, lanes 4, and 6), compared to that of naked DNAs (Fig. 2D, lines 3 and 5). The %protections from PmlI and BsiWI by a tetrasome on 601_185 bp were estimated to be 81.0 ± 6.3% (n = 3) and 93.6 ± 4.2% (n = 3), respectively. In contrast, when either naked or tetrasome assembled 601_185 bp was digested by NotI, both samples were completely digested (Fig. 2D lane 7-8). We concluded that the majority of tetrasomes was assembled near the center of 601 flanked by PmlI and BsiWI recognition sites (Fig. 2B).

Assessment of reconstituted nucleosomes

Next, we analyzed the assembled then purified nucleosomes, consisting of histones, H2A, H2B, H3, and H4. Four core histones and NAP1-coding mRNAs in the presence of 601_185 bp were used in a co-expression reaction for 6 h at 26 °C, followed by affinity purification. In the absence of core histone-coding mRNAs, no nucleosome nor DNA was detected (Fig. 3A, line 2). In the presence of all necessary components, the reconstituted nucleosome was determined (Fig. 3A, lane 3). The amount of reconstituted nucleosome prior to and after purification was estimated to be 201.2 ± 13.1 ng per reaction (n = 3) and 19.6 ± 1.6 ng per reaction (n = 3), respectively. Thus, ~10% of tetrasome was purified by affinity purification. Thus, the core histones-DNA complexes were formed in high purity. To assess if the nucleosomes had a canonical structure, that is, a DNA wrapping 1.7 times around core histones, a MNase assay was performed (Fig. 3B). After 10 min of MNase digestion, about 153 bp DNA fragment was observed (Fig 3B, lanes 3 and 4), consistent with the reported core nucleosomal DNA length [2,28]. When an excess amount of MNase was added to the nucleosome, most of the DNA wrapping around
histone octamer was diminished (Fig 3B, lane 5). Additionally, around 100-110 bp subnucleosomal DNA fragment was found after MNase digestion. Observed subnucleosomal DNA fragments from MNase digestion will be discussed later. From these results, we concluded that the purified nucleosome maintained an expected geometry.

**Fig. 2.** Analysis of assembled tetrasomes. (A) Reconstituted and purified tetrasomes were analyzed by 6% native polyacrylamide gels and visualized with ethidium bromide. Lane 1 indicates the naked 601_185 bp DNA. Lanes 2 and 3 show the formation of tetrasomes in the absence or presence of H3/H4 mRNAs in the co-expression assembly reaction followed by a His6-tag purification, respectively. (B) The cleavage sites of restriction enzymes, NotI, BsiWI, and PmlI on 601_185 bp are indicated. The filled triangle indicates the dyad of the Widom 601 sequence. (C) Scheme of restriction enzymes assays on the purified tetrasomes, in which PmlI and NotI were reacted at 37 °C, and BsiWI was incubated at 55 °C. (D) DNA fragments after restriction enzyme digestions were analyzed on 10% native PAGE. 601_185 bp derived from the purified tetrasomes in the absence of restriction enzyme is shown (lane 1). Naked 601_185 bp or tetrasome, indicated as \( \pm H3/H4 \), was digested with PmlI, BsiWI, and NotI.

**Fig. 3.** Reconstituted nucleosomes. (A) Assembled and purified nucleosomes were assessed by 6% native PAGE. Lanes 1-3 indicate the naked 601_185 bp, the reactions in the absence or presence of histone mRNAs in the co-expression reconstitution reaction, respectively. (B) Purified nucleosomes were digested by increasing amounts of micrococcal nuclease, 0, 0.08, 0.4, 1.6 U for 10 min at 37 °C. The samples were analyzed by a 3% agarose TBE gel and stained with ethidium bromide.

**Atomic force microscopy analysis of purified tetrasomes and nucleosomes**

Purified tetrasomes on 601_185 bp and nucleosomes on 601_307 bp (80bp non-nucleosome positioning sequences flank the Widom 601 sequence) were placed on an APS coated mica, and the topography of the samples was analyzed by AFM. To note, the reconstituted nucleosomes on 601_307 bp were purified and confirmed (Fig. S1). This construct was expected to add two ~ 80 bp free DNA ends from the nucleosome core particle under AFM measurement. Typical images of the tetrasome on 601_185 bp and nucleosome on 601_307 bp are shown in Fig. 4A and B (Fig. S2), respectively. From 100 each tetrasomes and nucleosomes, distributions of heights were analyzed (Fig. 4 C). The average and standard deviation of the tetrasomes were estimated to be 1.2 ± 0.4 nm (\( n = 100 \)), statistically significantly different from that of the nucleosomes with 2.7 ± 0.3 nm (\( n = 100 \)), \( P \)-value <0.001 (t-test). The diameters of the tetrasomes and nucleosomes were also distinguishable (\( P \)-value <0.001, t-test), 10.1 ± 1.8 nm and 16.5 ± 2.3 nm, respectively (Fig. 4D).

**Discussion**

Here, we reported a method to prepare purified tetrasomes and nucleosomes on the Widom 601 sequence under a NAP1-mediated co-expression assembly reaction, which could provide a preferable substrate for epigenetic enzyme screening (Fig. 1). The function of
NAP1 during a nucleosome assembly is suggested to prevent non-nucleosomal histone-DNA interactions [9,29,30]. Consistent with the previously reported chaperone-mediated tetrasome assembly using recombinant histones H3 and H4, we were able to assemble tetrasomes under a novel co-expression mode (Fig. 2A). It was suggested that there is only one tetrasome formed per short DNA sequence, and we assessed the position of tetrasomes on the 601_185 bp sequence using restriction enzymes (Fig. 2B-D). *NotI*, *BsiWI*, and *PmlI* recognition sites are located at −75 to −82, −3 to −8, and +49 to +54, relative to a dyad, respectively, and the region between −8 and +54 was indicated to be protected by a positioned tetrasome. In an earlier study, it was shown that the tetramer is the major determinant of the nucleosome positioning on the 208 bp sea urchin 5S rRNA sequence [31]. More recently, a sequence-dependent tetrasome positioning on the Widom 601 sequence was shown to be due to the high bendability and twistability of this sequence, which in turn stabilize the histone-DNA complex [30]. Thus, the reconstituted tetrasomes in this study using co-expression assembly are thought to be comparable to the tetrasomes assembled by salt gradient methods.

The purified reconstituted nucleosomes using NAP1-mediated co-expression reconstitution on 601_185 bp were also confirmed (Fig. 3A), and MNase assay was performed to determine the length of nucleosomal DNA (Fig. 3B). Around 150 bp DNA fragment was produced by a partial MNase digestion, suggesting that a canonical nucleosome was formed. Additionally, observed 100-110 bp fragments are thought to be derived from subnucleosomal species, which might be produced during the MNase digestion (Fig. 3A). It is known that the nucleosome core particle can be invaded by the aggressive nuclease activity of MNase, resulting in shorter...
DNA fragments [32]. Hence, observed 100–110 bp fragments were thought to be derived from the DNA wrapping around H2A/H2B/(H3/H4)_2 hexamer, consistent with reported notions [33–35].

Finally, purified tetrasomes and nucleosomes were analyzed by AFM, and the heights and diameters were measured (n = 100 each) (Fig. 4 and Fig. S2). The tetrasome on a 601_185 bp shown in Fig. 4A indicated that there are ~20 and ~14 nm free DNAs sticking out from the core particle to the direction toward 12- and 3-o’clock, respectively, which correspond to the expected free DNA length (bp) of ~60 and ~40 bp, respectively. Similarly, observed ~33 and ~22 nm free DNAs sticking out from the core particle to the 12- and 4-o’clock directions, respectively, are in accordance with the expected ~98 and ~65 bp of non-nucleosomal DNA on a 601_307 bp construct (Fig. 4B). However, the DNAs sticking out from the tetrasomes and nucleosomes were only seen in limited number of samples due to lack of resolution. The heights and diameters between the tetrasomes and nucleosomes were statistically different from each other (Fig. 4C). In agreement with the previous studies, the average height and diameter of nucleosomes measured in this study are around 2.7 and 16.5 nm, respectively (Fig. 4C and D) [36,37]. Moreover, the average diameter of the tetrasomes of around 10 nm is consistent with the previous report [37]. Of note, we were not able to compare the height of the tetrasomes measured in this study to others due to limited data describing the height of the tetrasomes acquired from the AFM image. Alternatively, we estimated the volume of observed tetrasomes in our analysis accordingly to the previous study [38]. The volume of reconstituted tetrasomes in our analysis was 49 ± 28 nm³ (n = 100), which is well within the range of the reported volumes of tetrasomes obtained by the molecular dynamics simulation [35].

In summary, we successfully reconstituted both tetrasomes and nucleosomes by using a wheat germ co-expression system in the presence of the Widom 601 sequence, followed by affinity purification. Both reconstituted tetrasomes and nucleosomes seemed stably positioned on the nucleosome positioning sequence; hence, they were successfully purified. The purified tetrasomes and nucleosomes using our method may allow us to understand the mechanisms of chromatin assembly and disassembly dynamics by evaluating the functions of chromatin assembly factors under near physiological condition. Furthermore, purified tetrasome and nucleosome should be favorable non-modified homogeneous substrates for downstream applications such as epigenetic enzyme screening [20]. In such case, a TEV protease can be used during affinity purification process, which will provide a tag-free N-terminal histone H4-containing tetrasomes or nucleosomes [22]. Overall, our current method has clear advantages over classical reconstitution methods [20] (i.e., assembled tetrasomes and nucleosomes in a co-expression reaction are in native state and they are free from typical epigenetic modifications). However, it should be stated here that there are several limitations. For example, the product yield is not comparable to the conventional methods, which utilize recombinantly expressed histones, unless we increase the reaction volume of the current protocol more than 100 times. Additionally, we do not know the atomic-level structure of our reconstituted tetrasomes and nucleosomes to date, so a large-scale reconstitution and subsequent structural determination such as X-ray crystallography or Cryo-EM are needed in future research.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

Data accessibility

The data that support the findings of this study are available from the corresponding author (takasuka@cen.agr.hokudai.ac.jp) upon reasonable request.

Author contributions

KO and TET designed the research. KO, SH, KM, and KU performed experiments. KO, PB, ST, and TET wrote the manuscript.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Purified reconstituted nucleosome on 601_307bp, analyzed by 6% PAGE.

**Fig. S2.** Overview of AFM images shown in Fig. 2. Tetrasome (A) and nucleosome (B) particles are shown with color-coded height (0–2.6 nm) (scale bar = 100 nm).