Study of NAP Adsorption and Assembly on the Surface of HOPG *

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ABSTRACT

NAP is an octapeptide that has demonstrated a neuroprotective/therapeutic efficacy at very low concentrations in preclinical studies and in a number of clinical trials. Yet little is known about its structural organization at low concentrations. Here, we have employed atomic force microscopy to investigate NAP peptide assembly on graphite in aqueous media at nanomolar concentration. High spatial resolution scans of NAP assemblies reveal their fine structure with clearly resolved single NAP units. This observation leads us to conclude that NAP molecules do not form complex self-assembled structures at nanomolar concentration when adsorbed on graphite surface.

Keywords: NAP; Atomic Force Microscopy; davunetide; adsorption; peptides

Introduction

NAP is an octapeptide with the amino acid sequence H_2N-L-Asn-L-Ala-L-Pro-L-Val-L-Ser-L-Ile-L-Pro-L-Gln-COOH [8, 17]. It is a peptide snippet from the activity-dependent neuroprotective protein (ADNP), [1], a protein essential for brain formation [20, 24] recently found to be mutated in autism [10], deregulated in schizophrenia [5, 21] and in patients with Alzheimer's disease [29]. In this respect, multiple preclinical evaluations, as well as clinical trials with NAP (davunetide) showed efficacy of the peptide [18, 19]. In particular, protection of cognitive scores in patients with mild cognitive impairment and daily living activities in schizophrenia patients were attributed to brain protection [12, 13].

There are two proline residues in the structure of NAP. The presence of these residues with their exceptional conformational rigidity prevents the NAP molecule from having a linear configuration (Figure 1). Thus unlike linear peptides, NAP should form neither a typical α-helix nor the less common β-sheet structure, as a primary step in molecular self-assembly. Surprisingly, little is known about the initial steps of NAP assembly and what structures they embrace, although it has been shown to form random structures when studied with circular dichroism in various solvents [9]. Interest in the early steps of NAP self-assembly in aqueous media is largely due to its bioactivity at very low (sub-nanomolar) concentrations. A hypothesis to explain this activity is the formation of self-assembled structures that interact further with target molecules, either losing activity at high concentrations, or having to concentrate at the binding point [3]. This report investigates, using atomic force microscopy (AFM), whether NAP self-assembles into such macromolecular nanostructures at low concentrations at a well-characterized liquid-solid interface. For the purpose of this work, we define macromolecular nanostructure as periodical structure assembled of single NAP peptides as opposed to random assembly. AFM has been used widely in investigations of self-assembled peptide structures [6, 27].

Experimental details

Samples were imaged with an AFM (Multimode 8 scanning probe microscope equipped with NanoScope V controller, BrukerNano, Coventry, UK) in tapping mode in ambient conditions. Standard silicon Multi75Al tips (Budget Sensors, Sofia, Bulgaria) with resonance frequencies and spring constants of about 75 kHz and 2.84 N/m respectively were used for imaging in ambient conditions. We have also utilized super-sharp diamond-like carbon (DLC) tips NSG01_DLC (NT-MDT, Zelenograd, Russia) with a nominal tip radius of ~1 nm and a spring constant of about 5.5 N/m.

Highly ordered pyrolytic graphite (NT-MDT, Zelenograd, Russia) was selected as a substrate to study peptide association in pure water. The surface of HOPG is made of layers of carbon atoms and thus is hydrophobic and provides no specific binding sites.

NAP (obtained from Allon Therapeutics Inc.) was dissolved in deionised water to a certain concentration. Freshly cleaved (top layers of graphite were mechanically removed with adhesive tape) HOPG surface was further exposed to solution. The HOPG surface was washed with 400 microL of fresh deionised water after adsorption to remove loosely bonded material. All samples were extensively dried in a N_2-stream before imaging. We typically performed imaging straight after the preparation.

All images were processed and analysed with WsXM software [11].

Results and Discussion

In the first instance we estimated the molecular dimensions of possible NAP conformations as this is important for the understanding of results obtained with AFM. Suitable low energy conformations of NAP were used to estimate the molecule's dimensions from a molecular structure built in ChemBio3D (Perkin Elmer, CambridgeSoft, UK). We carried out a standard optimization for a single molecule in vacuum at a semi-empirical level with the AM1 method as implemented in GAMESS [26]. We chose a conformation with a maximum number of intramolecular hydrogen bonds as a starting point. Although the NAP molecule will inevitably exhibit different conformations in solution, especially in aqueous media, we believe that when adsorbed on a hydrophobic graphite surface the model in vacuum could well approximate its conformation. The corresponding dimensions for the optimized conformation are shown in Figure 1 which represents a molecular model of NAP together with a Connolly molecular surface as estimated using ChemBio3D software package. The Connolly molecular surface represents the van der Waals surface as drawn by a test solvent sphere of 1.4Å (mimicking a water molecule) radius as it touches the van der Waals spheres of individual atoms. This optimization result suggests that there is potentially a system of multiple hydrogen bonds that twist the molecule into a pincer-like conformation with the following dimensions: 1.7 nm x 1.1 nm x 0.8 nm. Hence all hydrophobic alkyl groups form an exterior, with hydrophilic groups creating an interior part of the octapeptide molecule. The above considerations hold only when NAP molecules exist in solution and adsorb on a graphite surface as single molecules, i.e. at low concentrations.

Here we have used a 1 nM solution of NAP in deionised water (ddH_2O) to study its adsorption and possible subsequent assembly on HOPG. This was chosen as a test substrate as it provides an atomically flat, clean hydrophobic surface with minimal specific interactions, such as hydrogen bonding.

The absence of specific substrate-substance interactions helps to minimize any surface induced disruption of peptide assembly or undesired chemical bonding. We followed the assembly process with AFM at the HOPG interface over periods of up to 24 hours. At 24 hours the system was judged to have reached equilibrium by consideration of the layer growth. Example results are presented in Figure 2. We detected structures within the first hour of HOPG surface exposure to NAP solution in water: AFM revealed that NAP molecules form a fairly uniform layer with an average thickness of ~1.5 nm. We found that the layer consists of assembled nanostructures. We have further observed no major differences in the layer structure within 24 hour of its growth. This indicates that NAP molecules do not assemble into any specific macromolecular architecture at the given concentration or below. In fact, even at higher concentrations (up to 1mM) we have not observed the formation of any well-defined macromolecular structures.

We have carried out further detailed inspection of the NAP species after 1 hour adsorption to address their higher-ordered assembly (Figure 3). After 1 hour exposure to a NAP solution, the surface is predominately covered with small assemblies, reaching ~60nm in diameter and up to ~1.5 nm in height. Close inspection of these flat nanoassemblies reveals that they are composed of small nearly-round shaped units. We have not observed any periodicity in the way that these are assembled. The average height for a single unit is about ~1nm which given the expected surface-molecule separation of ~0.3-0.5 nm typically observed on HOPG [16] gives an estimated unit thickness of ~0.5-0.7 nm. This strongly suggests that the observed single unit is a single NAP molecule, if judged by its thickness. The observed size of units is in the range of 4-6 nm in diameter which is significantly larger than an estimated dimension for a pincer-like conformation of NAP molecule, which is about ~1.7 nm. These dimensions might therefore suggest lateral association of a number of NAP molecules. Another possible reason for increased dimensions is broadening of observed features caused by the geometry of tip apex as discussed at length in the literature [2, 28]. We have further investigated this possibility and the extent of broadening through the use of ultrasharp diamond probes. These probes have a nominal tip radius of <1nm and hence should provide improved spatial resolution. Such tips have previously been reported to produce true molecular resolution on polydiacetylene crystals whilst imaging in tapping mode in air [15]. The size of individual units observed using these tips was in the range of ~2 - 3 nm (Figure 3C-E). Therefore we clearly observe units close to the estimated dimensions of a single NAP molecule rather than several laterally associated molecules. Given some inevitable broadening of the features during AFM imaging [28], which we cannot unambiguously determine at such a small scale, we can conclude that we observe single NAP molecules. Slightly larger dimensions of some units are likely to be due to their hydration, as one would expect for a peptide molecule in an ambient environment. We have presented the

possible model of the NAP assembly based on the AFM findings (inset, Figure 3C). The model shows single NAP molecules in pincers-like conformation forming a nanoscale lateral assembly.

Providing our assumption is correct and that we observe assemblies of single NAP molecules, then due to the hydrophobic nature of the exterior, as discussed above, they should have weak intermolecular interactions. This assumption is supported by the ease of moving single NAP units while scanning as demonstrated on Figure 3E. The unit is highlighted with an arrow. It is worth noting that single units retain their dimensions during imaging and while being moved by the AFM tip. This indicates much stronger interactions within the single unit as compared to intermolecular forces.

These findings are consistent with the observed high potency for the activity of NAP *in vitro*. Original pharmacological studies [1], which were confirmed in versatile systems of neuroprotection [e.g. [30]], indicated activity at a broad concentration range, with the curious results of potency at femtomolar - nanomolar concentrations [7]. Together, our findings suggest that NAP molecules do not assemble to form large architectures; rather, there appears a low level of assembly (small clusters) of the peptide on graphite. Thus, NAP molecules may associate with neuroprotective targets either in a single molecular form or as a small molecular cluster, to fortify, for example, microtubules in nerve cells [22] and protect axonal transport [14, 25]. At the microtubule level, single NAP molecules may amplify protein interaction at the level of the growing tip of the microtubule[23]. Furthermore, small NAP clusters may be associated with membrane pore formation, which may enhance NAP cellular uptake [4].

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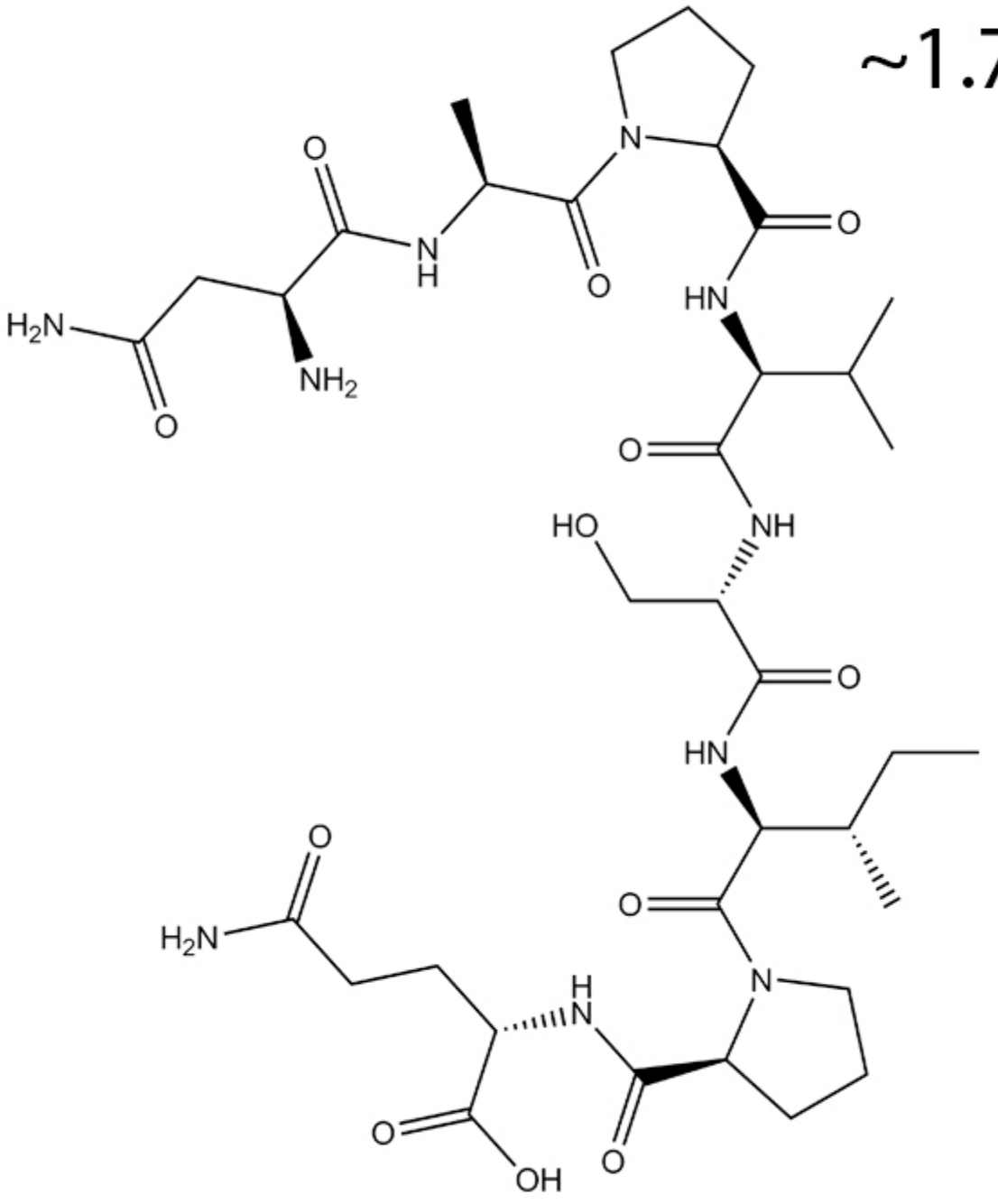
Figure 1. Optimized NAP structure and corresponding dimensions for the most stable conformation (pincer-like conformation). a – a model showing aminoacids sequence in NAP; b and c two projections of the stable conformation with Connolly molecular surfaces overlaid.

Figure 2. A set of AFM images showing NAP adsorption on HOPG surface at: a - 1h, b – 3h, c – 24h.

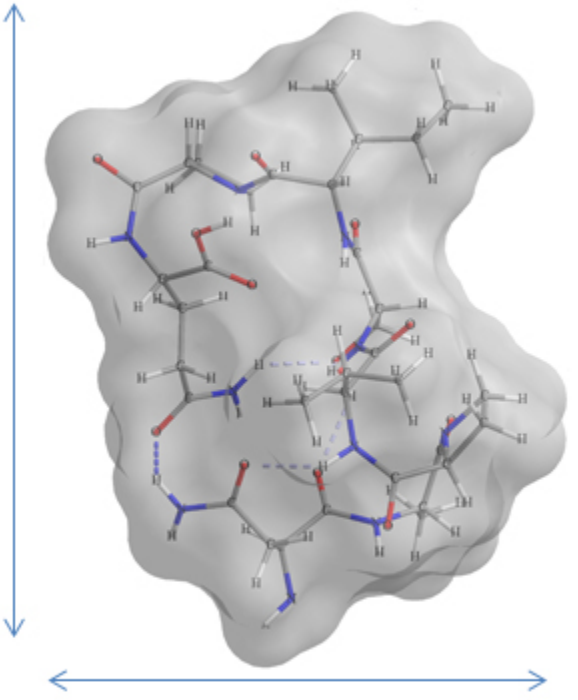
Figure 3.

High resolution images of NAP assemblies on a HOPG surface. Images a and b were acquired with Multi75Al Si-probe, c-e with an ultrasharp NSG01_DLC probe. The inset on image c shows a putative model for the NAP assembly on HOPG in pincer-like conformation; the blackened area represents a single unit observed in AFM images. The arrow on image e points at one of the units being detached during scanning from the same assembly shown on the image d, f – a crossection for the image e.

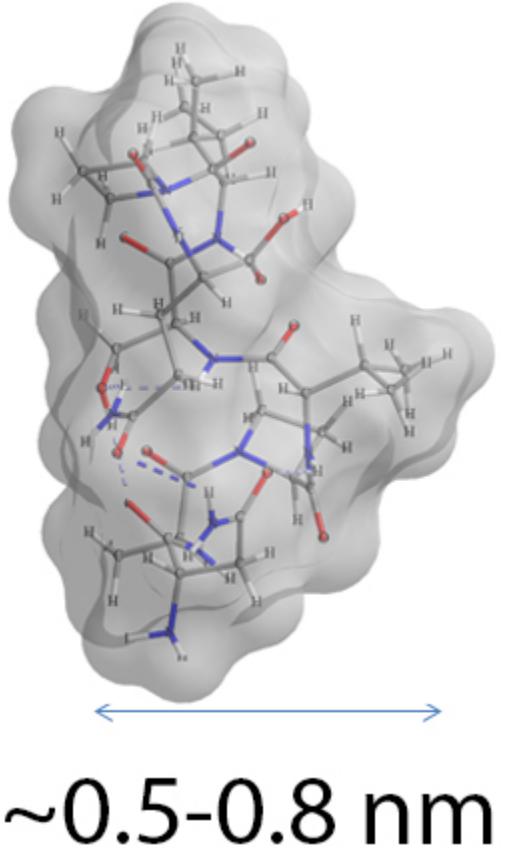
a

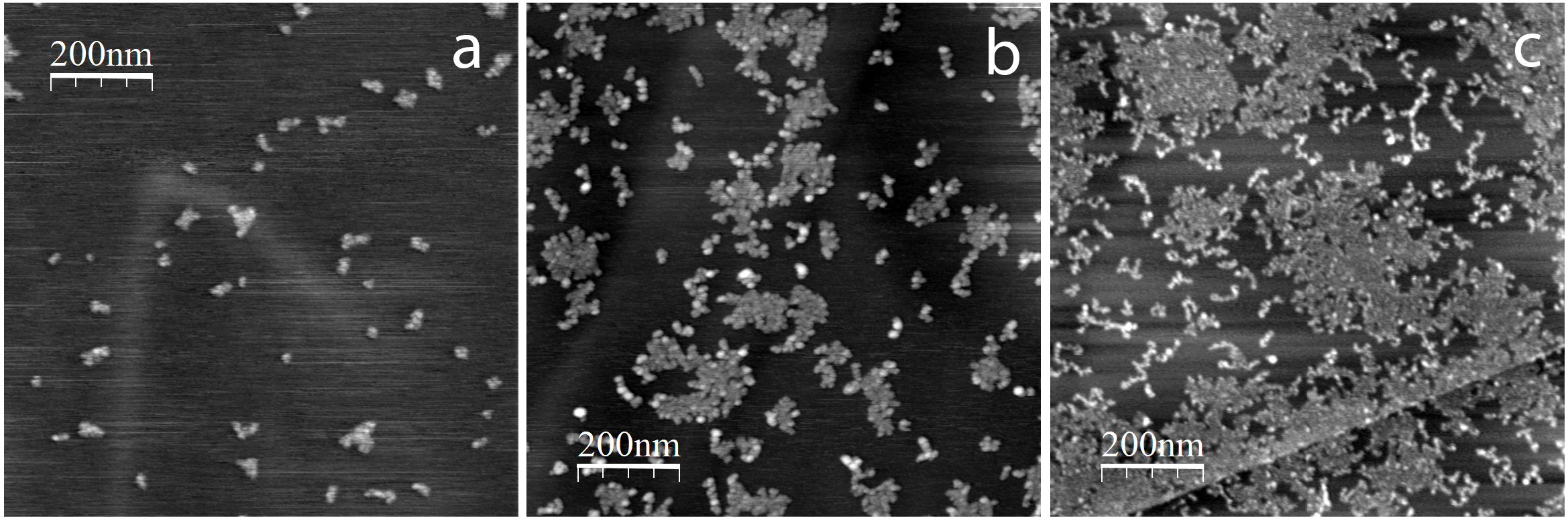


b



c




200nm
a
b
200nm
c
200nm

a

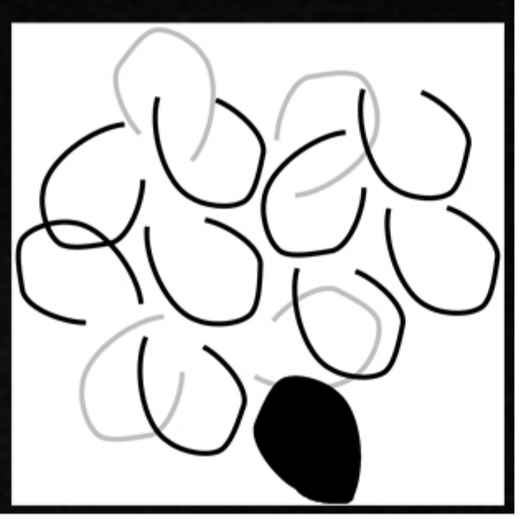
100nm

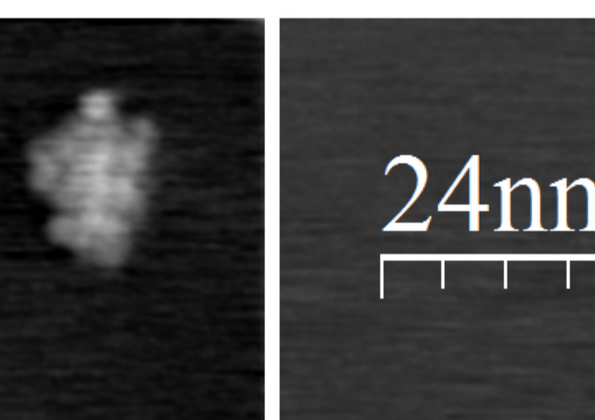
b

24nm

c

30nm





d

16nm

e

16nm

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