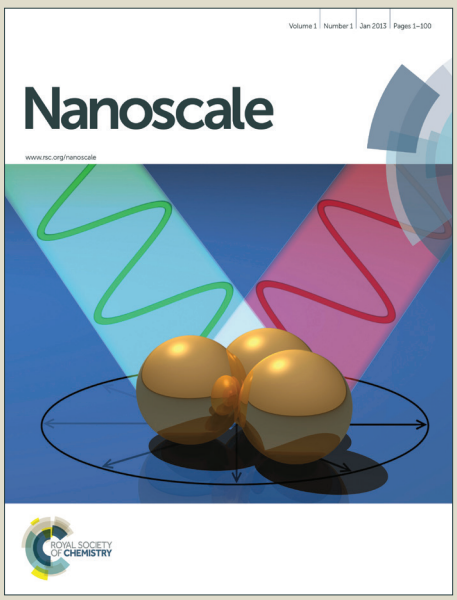
Nanoscale

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/nanoscale

Reply to the comment on "The Ultrastructure of Type I Collagen at Nanoscale: Large or Small D-Spacing Distribution?"

Thank Dr Wallace for his interest in our newly published paper[1], in which we found that the distribution of D-spacing in type I collagen was much smaller than that reported before. We appreciate Wallace's comment and are pleased to have the opportunity to present additional discussions about this work.

Wallace questioned about the effect of thermal drift on the measured D-spacing value. They performed an experiment in which a region in bone was continuously scanned over 1.5 h at 2 Hz (20 scans) to investigate the impacts of thermal drift. They found that the variation in D-spacing of individual fibrils (orientation not mentioned) was less than 2 nm. We also carried out a similar experiment. Two collagen fibrils on mica (in a 3 μm × 3 μm region) was scanned up and down continuously for over 2.4 h at 1 Hz (17 scans). For one collagen fibril with 29° with respect to the horizontal orientation, the variation in D-spacing was about 2 nm. For the other collagen fibril which was parallel to the horizontal orientation, the value of D-spacing remained stable. These results suggested that the thermal effect has influence on the measured D-spacing value. However, this influence is more obvious on the slow scanning axis, but is negligible on the fast scanning axis.

Since there is influence of thermal effect on the precise measurement, the distribution of the measured D-spacing value would be larger than the true value if this effect is not minimized. However, it seems that the 2-nm variation of D-spacing in single collagen fibrils is too small to be the reason of a large D-spacing distribution (over 10 nm) reported in Wallace and colleagues' work. We analyzed the D-spacing distributions of randomly oriented collagen fibrils, and found that the distribution of D-spacing values measured without minimizing the thermal effect was only 5.5 nm, which is still smaller than 10 nm.

We compared our experimental procedure with that from Wallace and colleagues. We found that a most likely reason might be the difference in collagen samples selected for statistic analysis. In our work, we mainly focused on single collagen fibrils which lay on the flat surface of mica, and selected the straight parts of the fibrils for measurement. However, the samples used in previous reports were tissue samples or collagen fascicles. In images of previous reports, it could be noticed that the surfaces of collagen fascicles were curved or not parallel to the surface of substrates[2]. When collagen samples with surface curvature were used for imaging, there are fewer measurement points defining its image than that is parallel to the x-y plane. Such measurements will enlarge the measured D-spacing distributions.

There is another difference between the samples used in our and Wallace's works. The preparation of the bovine collagen used in our work involved several treatment steps including using 100% alcohol for dehydration before storage. Those collagen samples were resuspended in distilled water for at least one hour before AFM imaging. In Wallace's works, alcohol treatment was not used. There is possibility that the different preparation procedures of collagen samples might lead to differences in results. Thus we prepared collagen samples

from mice tail following Wallace's method[3], and checked the D-spacing distributions of the collagen fibrils with AFM. A preliminary statistic analysis for 19 collagen fibrils showed that the D-spacing distribution was 1.5 nm, which was much smaller than that in Wallace's reports. However, the amount of collagen fibrils we checked was limited and further work must be needed.

We agree with Wallace that it is important to calibrate the instrument properly before measurement. In the comment he mentioned that calibration at the full x-y range would lead to error when scan size was reduced for collagen imaging. In addition to full x-y range calibration, we also carried out fine-tuning calibration for x-y plane following the instruction manual in our study. Thus, the instrumental error had been much reduced in our work when imaging the collagen fibrils in reduced scan sizes. Calibration with 100 nm standard, as suggested by Wallace, should also be a good calibrating method for precisely measurement in small region.

We used section analysis for calculating D-spacing, while Wallace considered 2D FFTs as a better method. The Bruker's AFM software (Nanoscope Analysis) provided both functions. We had compared the calculated D-spacing values from the two methods (21 collagen fibrils) and found the variations between the two methods were within 1.5%. Therefore, while 2D FFTs is a useful method for calculating D-spacing values, section analysis is useful and convenient as well.

We consider that the methodologies we used in our work are appropriate and we believe that further works are needed to clarify the difference of measured D-spacing value between single collagen fibrils and collagen fascicles. We appreciate Wallace's attention and helpful discussion to our work, and we believe that all communications and discussions will inevitably be helpful to promote the progress in this research area.

Hai-Nan Su, Bin-Bin Xie

State Key Laboratory of Microbial Technology
Shandong University, Jinan, China

1. H.-N. Su, L.-Y. Ran, Z.-H. Chen, Q.-L. Qin, M. Shi, X.-Y. Song, X.-L. Chen, Y.-Z. Zhang and B.-B. Xie, *Nanoscale*, 2014, **6**, 8134-8139.
2. M. Fang, E. L. Goldstein, A. S. Turner, C. Les, B. G. Orr, G. J. Fisher, K. B. Welch, E. D. Rothman and M. M. B. Holl, *ACS nano*, 2012, **6**, 9503-9514.
3. J. M. Wallace, Q. Chen, M. Fang, B. Erickson, B. G. Orr and M. M. B. Holl, *Langmuir*, 2010, **26**, 7349-7354.