

## Reliable Tools for Quantifying the Morphological Properties at the Nanoscale

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### Abstract

We have developed a fast, reliable and robust code to analyze size distributions and morphological properties of nanostructures. A segmentation algorithm, based on watershed transformation, has been implemented and tested. We have used consistent definitions to quantify the sphericity, roundness and roughness, which critically modify the physicochemical properties at the nanoscale. Finally, our approach has been applied to some example systems. We demonstrate that our code can be applied to guide the synthesis of nanoparticles, to distinguish dead from living bacteria and also to predict the reactivity of nanoparticles used for environmental remediation.

**Keywords:** Image analysis; Watershed transformation; Particle size distribution; Nanoparticles; Transmission electron microscopy; Fractal dimension

### Introduction

It is well known that the physicochemical properties of nanostructures strongly depend on their size, shape and surface characteristics [1]. Moreover, the controlled fabrication of nanostructures with desired morphologies is a subject of great interest in a wide variety of branches of nanotechnology [2,3]. By varying the length scale, we can distinguish three different quantities such as the sphericity, the roundness and the roughness, which describe the morphological characteristics of nanostructures. The sphericity is an overall measure of the shape of the object that quantifies how similar to a sphere it is, the roundness (or angularity) is related to the number of edges and how smooth or sharp they are, and finally at the smallest scale the roughness (or smoothness) quantifies the surface texture. Although there are clear definitions for these descriptors, as those given by the International Organization for Standardization (ISO), there is no agreement on their use and terminology. In many cases, the definitions have multiple versions, which depend on the preference of the author. The lack of consensus on which definitions should be used obstructs the research progress and makes it difficult to design strategies for improving the realization and performance of nanoscale devices. Among the techniques used to characterize nanoscale systems, the transmission electron microscopy (TEM) is one of the most powerful in resolving structural details. However, the TEM does not provide quantitative structural information and the development of image analysis techniques and approaches is required. Available free codes possessing user-friendly interfaces, like Icy [4], Pebbles [5] or ImageJ [6], already exist. However, sometimes it is necessary to add new subroutines to perform specific tasks and the problem becomes very tedious and, in most of the cases, impossible to solve. Therefore, it is very useful to have access to an entire library of

robust, open source and low-level functions that can be easily used to structure your programs and algorithms. In this work, we have developed a robust code that allows us to quantify the morphological characteristics of nanoscale systems. We have proposed simple definitions for sphericity, roundness and roughness in such a way that the results can be readily transferred from lab to lab. As the TEM provide us 2D-projections from 3D objects, the main assumption of our approach is that we deal with homogeneous and isotropic structures. Therefore, we are able to analyze the morphological properties of the nanostructures by investigating just their 2D-projection.

### Materials and Methods

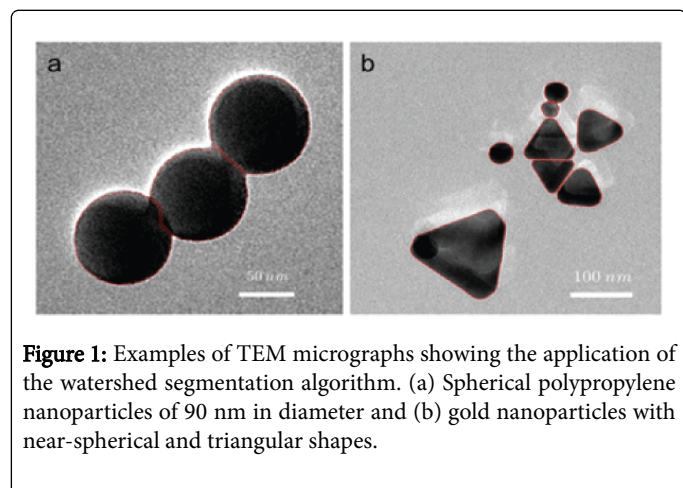
TEM imaging was carried out by using a FEI microscope, model Tecnai G2 spirit twin, operating at an accelerating voltage up to 200 kV. The samples were deposited in solution on a carbon-coated copper grid and gently dried with a lint-free tissue before performing the TEM measurements. As usual in microscopy techniques, the quality of the TEM images is largely responsibly of the final results. Therefore the image acquisition requires special attention [7]. It is important to note that the information extracted from TEM micrographs depends crucially on the magnification and brightness used during the measurements. To get an idea of the main morphological features low magnification micrographs are sufficient, while higher magnifications are required to extract the fine details. Together with the magnification, the brightness (or contrast) is largely responsible for the smooth of the nanostructure contours. In order to compare and quantify morphological changes, image acquisition should be performed with the same contrast conditions. Moreover, to analyze the roughness from different samples, the magnification is a critical parameter that should be maintained.

The image processing has been performed using a Macintosh with a 2.7 GHz 12-Core Intel Xenon E5 processor. The functions and

subroutines used in this work were written in Matlab and some algorithms require the Image Processing Toolbox.

## Results

The image processing process can be subdivided into three major subroutines: image preprocessing, image analysis and results interpretation. After loading the gray scale image, it is needed to edit the scale bar, which indicates to the program the conversion factor between pixels and lengths. This is performed by right-clicking on both ends of the scale bar and entering its length. Additionally, the optional crop function allows to remove parts of the image that are not of interest. The gray scale of the as-measured image ranges from 0 to 255, whereby 0 corresponds to black and 255 to white. The contrast of cropped images is enhanced by applying the *adapthisteq* function, which is based on the contrast-limited adaptive histogram equalization (CLAHE) method [8]. Once the contrast between the substrate and the structures of interest has been maximized, the grayscale image is converted into a binary image. The pixels having intensity values below and above a specific threshold range will be displayed as black and white (0 and 1) respectively. This process is automatically performed by using the Otsu's method [9]. Then, the areas smaller than a given limit, chosen by the user, will be removed and the edges of the binary shapes will be closed. Probably the most critical step of the image preprocessing is the segmentation. This process separates objects from the background as well as from each other. One of the most powerful approaches for solving image segmentation problems is the Watershed Transform. However, the direct application of the Watershed Transform usually produces an oversegmentation of the image. To overcome this issue we have used a common approach known as Minima Imposition [10]. This approach consists of overlapping a mask to the original gray scale image. Once the image has been segmented the contours of the closed areas are computed using the *bwboundaries* function. The *bwboundaries* function implements the Moore-Neighbor tracing algorithm modified by Jacob's criteria [11]. We have tested the segmentation algorithm with several images as those shown in Figure 1. As it can be observed the Watershed Transform is useful to separate touching nanoparticles but usually does not distinguish between overlapping objects. One of the most used approaches for achieving unsupervised segmentation of overlapping objects is done by predefining the shape of the particles. However, this approach limits the subjects of study to nanostructures of a certain shape [12].



**Figure 1:** Examples of TEM micrographs showing the application of the watershed segmentation algorithm. (a) Spherical polypropylene nanoparticles of 90 nm in diameter and (b) gold nanoparticles with near-spherical and triangular shapes.

The image analysis starts by computing the area and the perimeter for each closed area. This is performed through the *regionprops* and *bwboundaries* functions respectively. The areas cut by the image borders, i.e. those areas that are not completely inside the image, are excluded from the analysis by using the *imclearborder* function. Then, the centroid of each closed area as well as the equivalent, circumscribed and inscribed circles are computed. We have used an algorithm, based on the computation of the Feret's diameter [13] to determine the smallest rectangle enclosing each nanostructure. From the quantities described above we can calculate the sphericity and roundness, which have been defined as

Sphericity = Equivalent radius / Circumscribed radius and

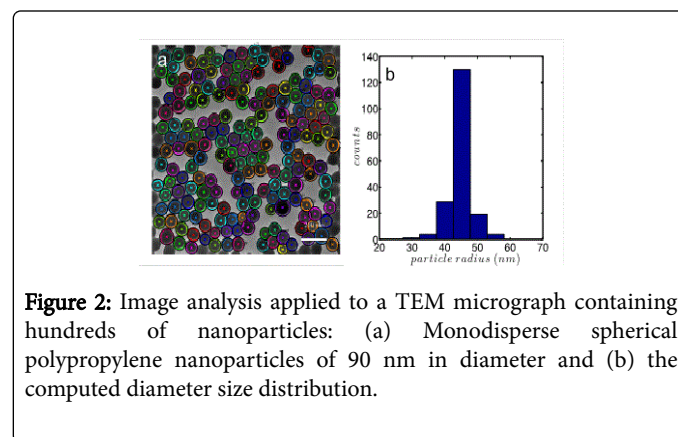
Roundness =  $4 \cdot \pi \cdot \text{Area} / (\text{perimeter})^2$ , respectively.

For the case of the roughness we have used two different definitions. The first definition, so-called Spherical Roughness, is useful to determine the roughness of spherical or spherical-like structures. In those cases, we have used the equivalent circle as base line from which the roughness is calculated as

Spherical Roughness =  $\frac{1}{L} \int_0^L |y(x) - c(x)| dx$ , where L is the perimeter of the equivalent circle, c(x) is the contour of the equivalent circle and y(x) is the contour of the particle. The second definition uses the fractal dimension as quantitative indicator of the complexity of the contour of nanostructures. We have used the box counting method [14] to define the fractal dimension. Different from the Spherical Roughness, the fractal dimension quantifies the roughness of an object by giving a number between 1 and 2, where 1 represents a smooth contour and 2 implies a high roughness.

## Discussion

For testing the working of our code we have analyzed several TEM images of different kind of nanostructures and briefly discussed some research problems, which evidence the utility of the morphological analysis. As a first example, we have analyzed an image containing up to several hundreds of mono-disperse spherical nanoparticles shown in Figure 2a. The particle radius distribution obtained from the colored particles is shown in Figure 2b. This kind of analysis can be useful to guide the synthesis of nanoparticles of controlled size and shape. For example, the ratio between the area of particle and the area of the circle and/or the rectangle that contain each closed area can be used to distinguish between triangular, hexagonal, square or circular shape.

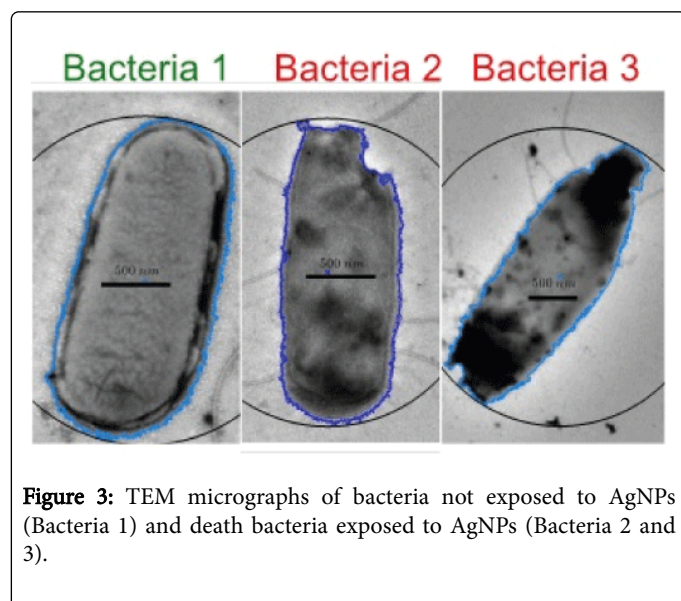


**Figure 2:** Image analysis applied to a TEM micrograph containing hundreds of nanoparticles: (a) Monodisperse spherical polypropylene nanoparticles of 90 nm in diameter and (b) the computed diameter size distribution.

The second example is related to the toxicity of silver ions and silver nanoparticles (AgNPs) towards bacteria and human cells, which has been extensively reported [15]. The presence of AgNPs causes the death of bacteria and results in a modification of its membrane. The amount of alive or dead bacteria can be easily quantified by analyzing the roughness of the bacterial membrane. Figure 3 shows typical TEM micrographs corresponding to live bacteria (Bacteria 1, in green) and dead bacteria (Bacteria 2 and 3, in red). Table 1 shows different morphological parameters corresponding to the bacteria shown in Figure 3. As it can be observed from the Table 1, dead bacteria show higher fractal dimension and smaller roundness as compared to live bacteria.

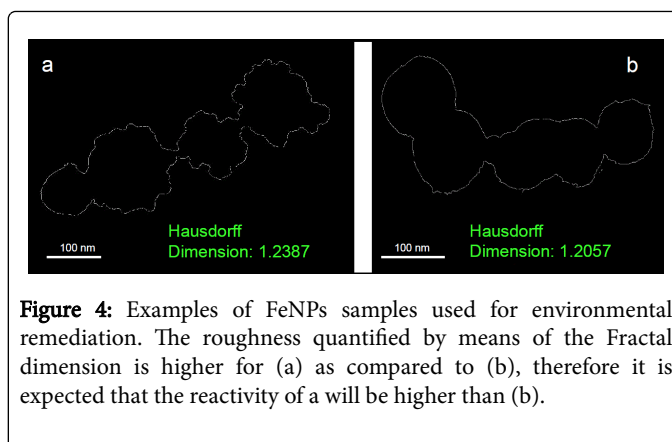
	Bacteria 1	Bacteria 2	Bacteria 3
Area (nm <sup>2</sup> )	1.6215 10 <sup>6</sup>	1.5101 10 <sup>6</sup>	2.3302 10 <sup>6</sup>
Perimeter (nm)	1.3788 10 <sup>4</sup>	1.1907 10 <sup>4</sup>	1.4908 10 <sup>4</sup>
Sphericity	0.6141	0.6322	0.5751
Roundness	0.1072	0.1338	0.1318
Fractal Dimension	1.3399	1.3456	1.3884

**Table 1:** Table of some morphological indicator obtained from the Bacteria images shown in Figure 3.



**Figure 3:** TEM micrographs of bacteria not exposed to AgNPs (Bacteria 1) and death bacteria exposed to AgNPs (Bacteria 2 and 3).

Finally, iron nanoparticles (FeNPs) have been applied for *in situ* environmental remediation of soil and water since long time ago [16]. The reactivity of nanostructures depends on the surface to volume ratio and therefore, it is expected that nanostructures possessing a higher roughness will be more reactive. In Figure 4 we show aggregates of FeNPs used for environmental remediation. The fractal dimension for the nanoparticles shown in Figure 4a is about 3.3% higher than for Figure 4b and accordingly, faster and more effective for remediation purposes. The synthesis of nanoparticles and novel materials used for environmental remediation could make use of the roughness as an indicator that predicts the remediation effectiveness.



**Figure 4:** Examples of FeNPs samples used for environmental remediation. The roughness quantified by means of the Fractal dimension is higher for (a) as compared to (b), therefore it is expected that the reactivity of a will be higher than (b).

## Conclusions

The image analysis is key tool towards the implementation of nanoscale systems in technological and biological applications. In this work, we have developed some fast and reliable algorithms to quantify the morphological characteristics of nanostructures. Moreover, we have demonstrated the utility of some indicators to address answers to various research problems e.g. optimization of synthesis methods, determination of toxicity of AgNPs and effectiveness of nanoparticles used for environmental remediation. Further work is in progress in order to extend the applications of the morphological analysis to newer biological, physical and chemical problems of interest.

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## References

- Albanese A, Tang PS, Chan WC (2012) The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annu Rev Biomed Eng* 14: 1-16.
- Daniel MC, Astruc D (2004) Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. *Chem Rev* 104: 293-346.
- Barth JV, Costantini G, Kern K (2005) Engineering atomic and molecular nanostructures at surfaces. *Nature* 437: 671-679.
- De Chaumont F, Dallongeville S, Olivo-Marin JC (2011). ICY: A new open-source community image processing software. *Biomedical Imaging: From Nano to Macro. IEEE International Symposium*, pp. 234-237
- Mondini S, Ferretti AM, Puglisi A, Ponti A (2012) Pebbles and PebbleJuggler: software for accurate, unbiased, and fast measurement and analysis of nanoparticle morphology from transmission electron microscopy (TEM) micrographs. *Nanoscale* 4: 5356-5372.
- Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. *Biophotonics international* 11: 36-42.
- Pyrz WD, Buttrey DJ (2008) Particle size determination using TEM: a discussion of image acquisition and analysis for the novice microscopist. *Langmuir* 24: 11350-11360.
- Reza AM (2004) Realization of the contrast limited adaptive histogram equalization (CLAHE) for real-time image enhancement. *Journal of VLSI signal processing systems for signal, image and video technology* 38: 35-44.

9. Fan JL, Zhao F (2007) Two-dimensional Otsu's curve thresholding segmentation method for gray-Level images. *Dianzi Xuebao (Acta Electronica Sinica)* 35: 751-755.
10. Dougherty ER, Lotufo RA (2003) Hands-on morphological image processing. SPIE Optical Engineering Press, Washington.
11. Gonzales RC, Woods RE, Eddins SL (2004) Digital image processing using MATLAB. Pearson Prentice Hall.
12. Shi J, Malik J (2000) Normalized cuts and image segmentation. *Pattern Analysis and Machine Intelligence, IEEE Transactions on Pattern Analysis and Machine Intelligence* 22: 888-905.
13. Xu R, Di Guida OA (2003) Comparison of sizing small particles using different technologies. *Powder Technology* 132: 145-153.
14. Liu JZ, Zhang LD, Yue GH (2003) Fractal dimension in human cerebellum measured by magnetic resonance imaging. *Biophys J* 85: 4041-4046.
15. Navarro E, Piccapietra F, Wagner B, Marconi F, Kaegi, R, et al. (2008) Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. *Environmental science & technology* 42: 8959-8964.
16. Zhang WX (2003) Nanoscale iron particles for environmental remediation: an overview. *Journal of Nanoparticle Research* 5: 323-332.

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