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Short Communication

Electron microscopy broadens the horizons of toxicology: The role of nanoparticles vehiculated by bacteria

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ABSTRACT

This communication shows the ways in which nanoparticles, originated by the biodestruction of plastic materials carried out by microbes, are able to access human organs by means of microorganisms acting as facilitators. The aim of this work is to highlight how electron microscopy is a fundamental and versatile technique of high value in the investigation of the interaction between bacteria and polymeric materials.

KEYWORDS: nanoparticles, polyurethane, prostheses, *S. aureus*, biodestruction, endocytosis, electron microscopy, bacteria-host cell interaction, infection, toxicology

INTRODUCTION

Biodestruction [1] can occur, for instance, in the oral cavity [2], where bacteria such as *Staphylococcus aureus* [3, 4] organized in microcolonies embedded in the biofilm [1], attack polymeric polyurethane (PU) prostheses [5] generating micro- and nano-sized debris [6, 7]. Electron microscopy is a fundamental and versatile technique of high value [8-14] in the investigation of the interaction between bacteria and polymeric materials [15]. In particular it can be of help since NPs are capable of interaction with the cellular machinery and are transported by active processes [16]; moreover NPs have other ways of interaction with cells, since in biological media, proteins and other biomolecules can arrange themselves in layers on the NPs' surface (protein corona). The corona is stable for time periods [17] similar to or longer than the typical time scale of the NPs uptake into the cells. Thus this is really "what the cell sees" [18, 19], with the protein corona acting as a "Trojan horse" that hides the NP [18].

The understanding of what controls the interactions at the interface between NPs and cells is essential to be able to exploit the potential of NPs for applications in nanomedicine [18, 20-22]. NPs may gain access to the body by inhalation, ingestion, intravenous administration, via skin absorption, or from prostheses generating debris [19, 22-27]. NPs can also be structured as nanofibers, thus introducing mechanical processes to the cell-NP interactions [28].

NPs based on materials such as natural or synthetic polymers, lipids and metals have received considerable interest as drug delivery vehicles [29, 30] or possible sources of toxic effects [25, 30-32], although as yet there is limited evidence of hazards [16, 33-35], and hence should be supplemented by the precautionary principle [36-38].

We will not consider any kind of engineered NP, but will focus on nanosized debris generated from the biodestruction of PU dental prostheses, the

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internalization of NPs by bacterial cells [15] and the subsequent invasion of host cells by NP-loaded bacteria.

The augmentation of the number of bacterial cells and the consequent depletion of nutrients together with the bacterial metabolic activity, that cause an increment of the acidity in the environment, damage the PU, generating NPs [7, 39]. They can interact with biological systems, move inside the body, reach the bloodstream and organs and can persist in the body for a very long time [23, 40, 41]. The effects of the NPs depend on their shape, size, surface characteristics and inner structure [16, 23, 42]. They can remain free or group together depending on the attractive or repulsive interaction forces between them [40, 43, 44], that can be affected when NPs move in a biological medium enveloped in the protein corona [17-19].

Nanosized particles have different characteristics compared to bigger ones while and when absorbed by an organism [23, 45]. This, together with the bioaccumulation associated with different rates of uptake and exit dynamics [16], can enhance the possible toxicity of nanosized materials. The typical time scale of NPs uptake, as well as the protein corona time stability, is in the order of hours [18]. Several reports show that NPs of 20-50 nm are taken up more rapidly than smaller or larger particles [45].

For particles with a positive charge will bind to the negatively charged cell surface, one would expect positively charged particles to be endocytosed [26, 46] more efficiently than negatively charged particles, influencing endocytic pathways (endocytosis, phagocytosis and pinocytosis) based on the formation of intracellular vesicles following invagination of the plasma membrane [23, 47].

It has been shown that PU micelles can act as an engineered drug deliverer [29]. Bulk PU is not toxic [48] but possible toxic effects can be associated to the small size of the particles [23], to the presence of the protein corona [18] and to the bioaccumulation [16] (ruled by the uptake time [16, 17, 19] and by the presence or absence of transport backward to the cell surface [16, 47]).

MATERIALS AND METHODS

In our experiment PU was incubated with *S. aureus* from 1 to 45 days at 37 °C. Sample preparation for electron microscopy is discussed by Didenko *et al.* [7, 15].

Transmission Electron Microscope and Scanning Transmission Electron Microscope images were obtained with a Tecnai F20 X-TWIN microscope (FEI Company, USA) equipped with a 200 kV FEG column and a CCD detector. In addition to TEM and SEM images, STEM technique was used and Bright Field, Dark Field and High Angle Annular Dark Field images were collected for better contrast and resolution and to gain more information on PU NPs' size and relative position compared to cell structures. Samples were analyzed in dual beam FIB/SEM Quanta 200 3D (FEI Company, USA) in both high and low vacuum, mostly at 5 kV electron beam acceleration.

RESULTS

FIB/SEM images show the bacterial bioadhesion to the plastic material, the subsequent biofilm formation, the nanosized debris generation (Fig. 1) and their attachment to the cell surface (Fig. 2). TEM Bright Field image shows that bacteria are able to internalize one or more NPs (Fig. 3). A detailed analysis of this image and similar ones [15] shows different steps of the internalization process (endocytosis) [26, 46]. PU NPs (whose size is less than 10 nm) appear enclosed in round membranous structures (vesicles) whose diameter is approximately 30 nm (Fig. 3).

TEM Dark Field image proves that bacteria are viable since typical features of bacterial reproduction through fission are visible even after long incubation time with the plastic material [49]. Therefore it can be said that the NPs internalization does not affect *S. aureus* viability (Fig. 4).

DISCUSSIONS

From our *in vitro* experiments and from literature [1, 7, 15], it is known that planktonic cells detach themselves from the biofilm. Hence it can be expected that *in vivo* bacterial nomad cells containing PU NPs contribute to the dissemination of the nanomaterial while spreading the infection to tissues.

S. aureus is known to be able to escape immune system surveillance [50-52] and to invade a number of organs through systemic dissemination provoking infections [53]. Moreover these bacteria after

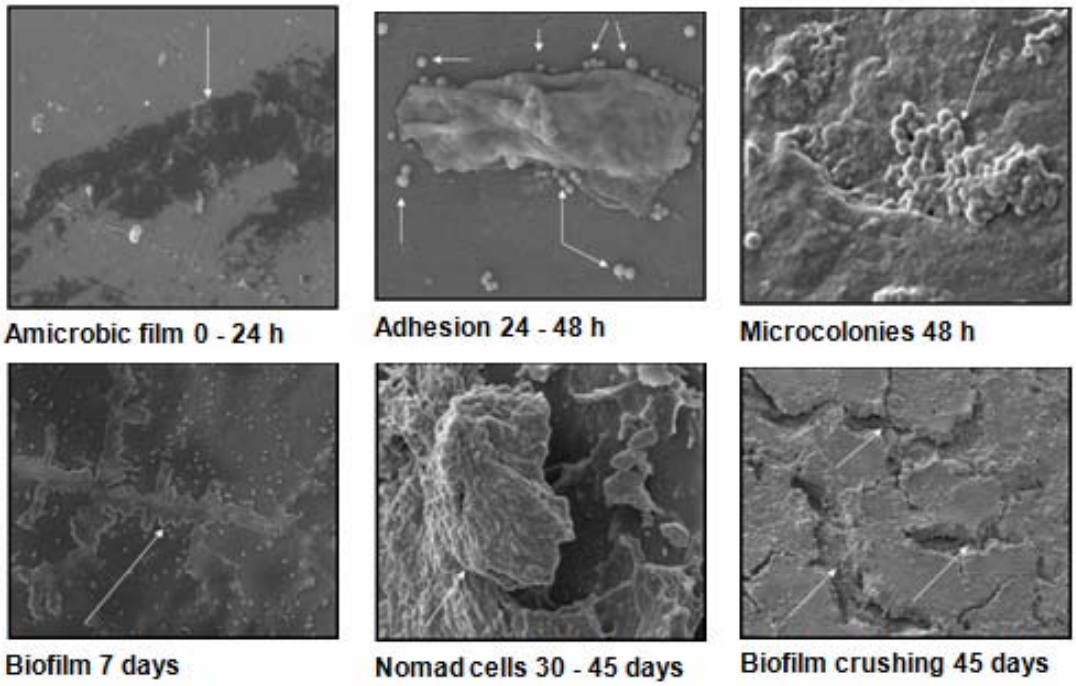

Fig. 1. FIB/SEM images at different magnifications show the fundamental steps of the interaction between *S. aureus* and PU: amicrobic film, bioadhesion, microcolonies formation, biofilm, nomad cells and biofilm crushing.

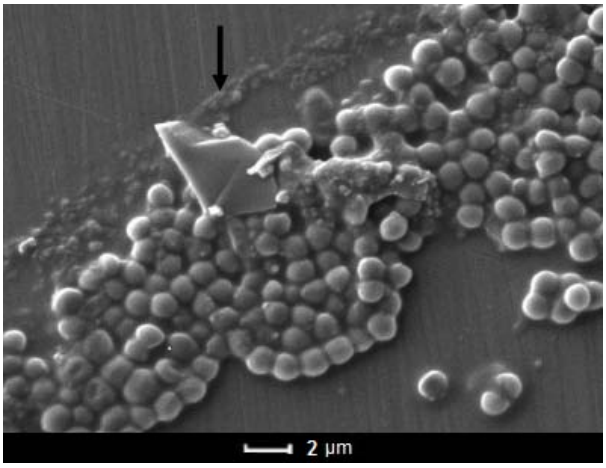

Fig. 2. SEM image of slices and fine grains of PU in bacterial mass.

attachment and colonization on host surfaces can eventually invade organ cells promoting their apoptotic processes and necrosis of the tissue [54, 55].

This allows microorganisms loaded with PU NPs, viable after the NPs internalization [49], to act as targeted vectors, vehiculating the NPs to the places

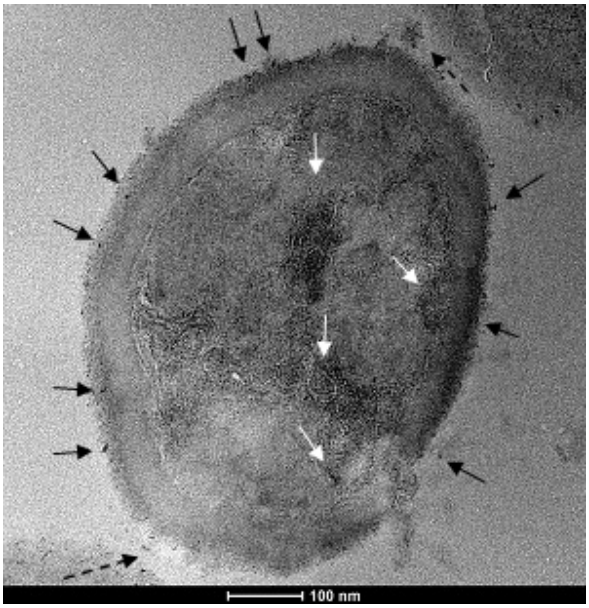

Fig. 3. TEM image of *S. aureus* after incubation with PU. PU particles can be observed on the cell wall (black ↑), inside the cell surrounded by membranes (white ↑) and in the external environment, in the proximity of the cell wall (black dashed ↑).

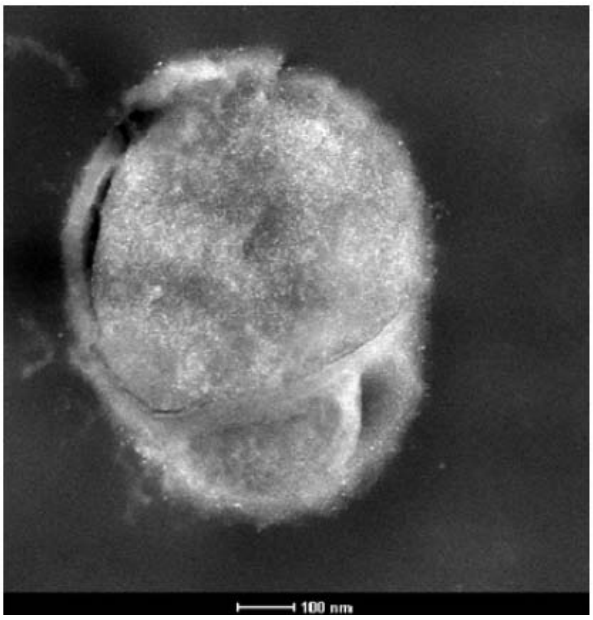

Fig. 4. TEM image of *S. aureus* fission proves that the cell viability is not compromised by the NPs internalization.

(host organ cells) that will become the infection loci. NPs spread in an efficient and oriented way reaching different body districts and, being hidden within the microorganisms, can move in the body without stimulating host innate and adaptive immune responses [53, 56, 57] (Fig. 3).

To sum up, our focus is on the PU NPs uptake by bacterial cells. Bacteria-tissue interaction in the infection processes [53] leads us to the conclusion that bacterial cells are an active and selective vehicle for NPs dissemination and cellular uptake. Therefore intracellular transport, recycling of NPs to the cell exterior, disturbance of cellular functions and metabolism of NPs both in bacterial and host cells have to be re-discussed.

S. aureus when internalized in the host cells (protected by phagocytes or linked to internal membranes and cytoskeleton [53, 58]) has three outcomes:

- reproduction, by which the number of bacterial cells increases, with one or more cells containing NPs;
- release of NPs inside the host cell through exocytosis [59], with NPs losing their enveloping membrane;
- natural or specific antibiotic induced death, with the consequent membrane lysis and the release of the NPs, still inside the vesicles, within the host cell.

Therefore in the host cell it is possible to find:

- PU NPs not surrounded by a membrane (free NPs) derived from exocytosis [59], although some researchers conclude that the import processes are irreversible and hence the export processes are absent [16]. NPs uptake and biodistribution are even more complex since endocytosis and exocytosis are influenced by the protein corona [16, 18, 42, 59] and by the cytoskeleton activity [58, 60];
- NPs not surrounded by a membrane (likely shielded by the protein corona) attached to the bacterial outer surface, therefore carried into the host cell by the bacteria themselves (Fig. 3);
- NPs shielded by vesicles.

Thus NPs behave differently according to their interface with the surrounding medium. Free NPs can display their toxic potential unless neutralized by phagocytosis. This endocytic process is influenced by the NPs effective charge that can be modified by the protein corona [61]. The membrane bound vesicles uptake by host cells allows the NPs to strictly interact with the cells' internal structures [61], specifically with the mechanisms involved in the vesicles' trafficking [47], and to escape phagocytosis. In both cases NPs coating (membrane vesicles or protein corona) delays immune (phagocytic) clearance of foreign particles [60].

CONCLUSIONS

In conclusion, the TEM, FIB/SEM and STEM ensemble, spanning over a wide range of magnifications and associated to different preparation methods, points to the existence of a complex NPs non-diffusive strategy by giving shots of different steps, such as NPs production, release and spreading driven by infection committed bacteria. This provides new opportunities in nanomedicine and gives evidence of unexplored processes responsible for the NPs toxicity that necessitate careful consideration from the point of view of nanomedical safety issues [16, 47].

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ABBREVIATIONS

NP, nanoparticle; PU, polyurethane; TEM, Transmission Electron Microscope; SEM, Scanning Electron Microscope; FIB/SEM, Focused Ion Beam/Scanning Electron Microscope

REFERENCES

1. Ghannoum, M. A. and O'Toole, G. A. (Eds.), 2004, Microbial Biofilms, Amer. Society for Microbiology Press, Washington, DC, USA.
2. Pye, A. D., Lockhart, D. E. A., Dawson, M. P., Murray, C. A. and Smith, A. J. 2009, Journal of Hospital Infection, 72, 104.
3. Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I. and Dewhirst, F. E. 2005, Journal of Clinical Microbiology, 43, 5721.
4. Smith, A. J., Jackson, M. S. and Bagg, J. 2001, Journal of Medical Microbiology, 50, 940.
5. Bhola, R., Bhola, S. M., Liang, H. and Mishra, B. 2010, Trends Biomater. Artif. Organs, 23, 129.
6. Zachinyaev, Ya. V., Miroshnichenko, I. I. and Zachinyaeva, A. V. 2009, Russian Journal of Applied Chemistry, 82, 1321.
7. Didenko, L. V., Avtandilov, G. A., Shevlyagina, N. V., Smirnova, T. A., Lebedenko, I. Y., Tatti, F., Savoia, C., Evans, G. and Milani, M. 2012, Current Microscopy Contributions to Advances in Science and Technology, A. Méndez-Vilas, (Ed.) Formatex Research Center Publishing, 323.
8. Mestres-Ventura, P. 2007, Imaging & Microscopy, 9, 44.
9. McIntosh, J. R. 2001, The Journal of Cell Biology, 153, F25.
10. Wallace, P. K., Arey, B. and Mahaffee, W. F. 2011, Micron, 42, 579.
11. Candia Carnevali, D. and Milani, M. (Eds.), 2010, Proceedings of Summer School 2008 & 2009, Miriam, Società Editrice Esculapio, Bologna, Italy.
12. Milani, M., Drobne, D. and Tatti, F. 2007, Modern Research and Educational Topics in Microscopy, A. Méndez-Vilas and J. Díaz (Eds.), Formatex Research Center Publishing, 787.
13. Milani, M., Drobne, D., Drobne, S. and Tatti, F. 2006, An atlas of FIB/SEM in soft materials and life sciences, Aracne Publishing, Rome.
14. Heymann, J. A. W., Hayles, M., Gestmann, I., Giannuzzi, L. A., Lich, B. and Subramaniam, S. 2006, Journal of Structural Biology, 155, 63.
15. Didenko, L. V., Avtandilov, G. A., Shevlyagina, N. V., Shustrova, N. M., Smirnova, T. A., Lebedenko, I. Y., Curia, R., Savoia, C., Tatti, F. and Milani, M. 2013, Open Journal of Medical Imaging, 3, 69.
16. Salvati, A., Åberg, C., Dos Santos, T., Varela, J., Pinto, P., Lynch, I. and Dawson, K. A. 2011, Nanomedicine: Nanotechnology, Biology and Medicine, 7, 818.
17. Walczyk, D., Baldelli Bombelli, F., Monopoli, M. P., Lynch, I. and Dawson, K. A. 2010, J. Am. Chem. Soc., 132, 5761.
18. Wang, F., Yu, L., Monopoli, M. P., Sandin, P., Mahon, E., Salvati, A. and Dawson, K. A. 2013, Nanomedicine: Nanotechnology, Biology and Medicine, 9, 1159.
19. Lynch, I., Salvati, A. and Dawson, K. A. 2009, Nature Nanotechnology, 4, 546.
20. Roiter, Y., Ornatska, M., Rammohan, A. R., Balakrishnan, J., Heine, D. R. and Minko S. 2008, Nano Letters, 8, 941.
21. Miller, G. 2012, Science, 336, 286.
22. Šimundić, M., Drašler, B., Šuštar, V., Zupanc, J., Štukelj, R., Makovec, D., Erdogmus, D., Hägerstrand, H., Drobne, D. and Kralj-Iglič, V. 2013, BMC Veterinary Research, 9, 7.
23. Revell, P. A. 2006, Nanotechnology Perceptions, 2, 283.
24. Buzea, C., Pacheco Blandino, I. I. and Robbie, K. 2007, Biointerphases, 2, MR17.
25. Nemmar, A., Holme, J. A., Rosas, I., Schwarze, P. E. and Alfaro-Moreno, E. 2013, BioMed Research International, 2013, Article ID 279371.
26. Kettiger, H., Schipanski, A., Wick, P. and Huwyler, J. 2013, International Journal of Nanomedicine, 8, 3255.
27. Genina, E. A., Bashkatov, A. N., Dolotov, L. E., Maslyakova, G. N., Kochubey, V. I., Yaroslavsky, I. V., Altshuler, G. B. and Tuchin, V. V. 2013, Journal of Biomedical Optics, 18, 111406.
28. Millaku, A., Drobne, D., Torkar, M., Novak, S., Remškar, M. and Pipan-Tkalec, Ž. 2013, Journal of Hazardous Materials, 260, 47.
29. Yu, S., He, C., Ding, J., Cheng, Y., Song, W., Zhuang, X. and Chen, X. 2013, Soft Matter, 9, 2637.

30. Zhang, X., Dong, Y., Zeng, X., Liang, X., Li, X., Tao, W., Chen, H., Jiang, Y., Mei, L. and Feng, S. 2014, Biomaterials, 35, 1932.
31. Alfaro-Moreno, E., Nawrot, T. S., Nemmar, A., Rosas, I. and Schwarze, P. 2013, BioMed Research International, 2013, Article ID 642974.
32. Danhier, F., Ansorena, E., Silvia, J. M., Coco, R., Le Breton, A. and Preat, V. 2012, J. Control. Release, 161, 505.
33. Bengalli, R., Mantecca, P., Camatini, M. and Gualtieri, M. 2013, BioMed Research International, 2013, Article ID 801214.
34. Simkó, M. and Mattsson, M. 2010, Particle and Fibre Toxicology, 7, 42.
35. Gatti, A. M. 2004, Nanopathology - The role of micro- and nano-particles in biomaterial-induced pathology, A RTD PROJECT QLRT-2001-147 (2002-2005) funded by European Commission.
36. UK-ILGRA (United Kingdom - Inter Departmental Liaison Group on Risk Assessment), The Precautionary Principle: Policy and Application, 2002.
37. Colvin, V. L. 2003, Nature Biotechnology, 21, 1166.
38. Hoet, P. H. M., Nemmar, A. and Nemery, B. 2004, Nature Biotechnology, 22, 19.
39. Gemmell, C. G. 1986, Journal of Medical Microbiology, 22, 285.
40. SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks), 2006, The appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies.
41. Thorpe, B. Nanoparticles in your cosmetics and consumer products: untested, unlabeled and of concern, Breast Cancer Action Montreal, http://www.bcam.qc.ca/content/nanoparticles-your-cosmetics-and-consumer-products-untested-unlabeled-and-concern
42. Lundqvist, M., Stigler, J., Elia, G., Lynch, I., Cedervall, T. and Dawson, K. A. 2008, Proc. Natl. Acad. Sci. USA, 105, 14265.
43. Gratton, S. E. A., Ropp, P. A., Pohlhaus, P. D., Luft, J. C., Madden, V. J., Napier, M. E. and DeSimone, J. M. 2008, Proc. Natl. Acad. Sci. USA, 105, 11613.
44. Mantecca, P., Moschini, E., Gualtieri, M., Longhin, E., Fascio, U. and Camatini, M. 2010, Abstracts/Toxicology letters, 196S, S37.
45. Jiang, W., Kim, B. Y. S., Rutka, J. T. and Chan, W. C. W. 2008, Nat. Nanotechnol., 3, 145.
46. Doherty, G. J. and McMahon, H. T. 2009, Annu. Rev. Biochem., 78, 857.
47. Iversen, T. G., Skotland, T. and Sandvig, K. 2011, Nano Today, 6, 176.
48. Howard, G. T. 2011, Recent Developments in Polymer Recycling, A. Fainleib and O. Grigoryeva (Eds.), Transworld Research Network Publishing, Kerala, India, 215.
49. Tzagoloff, H. and Novick, R. 1977, J. Bacteriol., 129, 343.
50. Carryn, S., Chanteux, H., Seral, C., Mingeot-Leclercq, M. P., Van Bambeke, F. and Tulkens, P. M. 2003, Infect. Dis. Clin. N. Am., 17, 615.
51. Clement, S., Vaudaux, P., Francois, P., Schrenzel, J., Huggler, E., Kampf, S., Chaponnier, C., Lew, D. and Lacroix, J. S. 2005, The Journal of Infectious Diseases, 192, 1023.
52. Sendi, P., Rohrbach, M., Graber, P., Frei, R., Ochsner, P. E. and Zimmerli, W. 2006, Clin. Infect. Dis., 43, 961.
53. Lowy, F. D. 2000, Trends in Microbiol., 8, 341.
54. Kuehn, M. J. and Kesty, N. C. 2005, Genes Dev., 19, 2645.
55. MacDonald, I. A. and Kuehn, M. J. 2012, Res. Microbiol., 163, 607.
56. Hornef, M. W., Wick, M. J., Rhen, M. and Normark, S. 2002, Nat. Immunol., 3, 1033.
57. Rooijakkers, S. H. M., van Kessel, K. P. M. and van Strijp, J. A. G. 2005, Trends in Microbiology, 13, 596.
58. Skruzny, M., Brach, T., Ciuffa, R., Rybina, S., Wachsmuth, M. and Kaksonen, M. 2012, Proc. Natl. Acad. Sci. USA, 109, E2533.
59. Dombu, C. Y., Kroubi, M., Zibouche, R., Matran, R. and Betbeder, D. 2010, Nanotechnology, 21, 355102.
60. Rodriguez, P. L., Harada, T., Christian, D. A., Pantano, D. A., Tsai, R. K. and Discher, D. E. 2013, Science, 339, 971.
61. Doorley, G. W. and Payne, C. K. 2012, Chem. Commun. (Camb.), 48, 2961.