A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition Chemie

GDCh

www.angewandte.org

Accepted Article

Title: A synthetic getaway biomimetic strategy for cytoplasm particle invasion

Authors: Monica L Fanarraga, Nerea Iturrioz-Rodriguez, Elena González-Domínguez, Eloisa González-Lavado, Laura Marín-Caba, Belén Vaz Araújo, Moisés Pérez-Lorenzo, and Miguel Correa-Duarte

To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201707769
Angew. Chem. 10.1002/ange.201707769

Link to VoR: http://dx.doi.org/10.1002/anie.201707769
http://dx.doi.org/10.1002/ange.201707769

WILEY-VCH

10.1002/anie.201707769

WILEY-VCH

COMMUNICATION

A synthetic getaway biomimetic strategy for cytoplasm particle invasion

N. Iturrioz-Rodríguez[#[a]], E. González-Domínguez[#[b]], E. González-Lavado[[a]], L. Marín-Caba[[b]], B. Vaz[[b]] M. Pérez-Lorenzo[[b]], M. A. Correa-Duarte*[[b]] and M. L. Fanarraga*[[a]]

Abstract: *The translocation of nanomaterials or complex delivery systems into the cytosol is a main challenge in nanobiotechnology. After receptor-mediated endocytosis, most nanomaterials are sequestered undergoing degradation, therapy inactivation or exocytosis. Here we explore a novel surface particle-coating made of adsorbed carbon nanotubes that provides coated materials with new properties that reproduce the viral cell invasive mechanisms, namely: receptor mediated endocytosis, endo-lysosomal escape and cytosolic particle release preserving intact cell viability. This novel biomimetic coating design will enable the intracytoplasmic delivery of many different functional materials endowed with therapeutic, magnetic, optical or catalytic functionalities thus, opening the door to a wide array of chemical and physical processes within the cytosolic or nuclear domains, and supporting the generation of new developments in the biotechnological, pharmaceutical and biomedical industries.*

Probably the biggest challenge in biotechnology and medicine is the design of versatile tools for targeted intracellular delivery of *ad hoc* combinations of materials and bioactive molecules for cell manipulation. Current nanodevices offer limited solutions to the cytoplasmic delivery problem of active compounds, being the biggest challenges: (i) specific cell recognition, (ii) preservation of the cargo from the hostile lysosomal chemical conditions, (iii) non-lethal endo-lysosomal (E-L) escape, (iv) carriage of different particles or chemicals into the cytosol, and finally, (v) clearance of the delivery vectors to avoid long-term cytotoxicity.

To date, there is only partial understanding of how to control the intracellular fate of the carrier systems after endocytosis. Most delivery vectors get trapped in the E-L vesicles, where the hostile enzymatic and chemical conditions destroy the nanomaterials and/or inactivate the therapies.[1,2] Thus, there is a need for effective strategies triggering viable synthetic E-L membrane translocation.[3]

In nature, viruses can penetrate into the cytosol after receptor-endocytic uptake preserving intact cell viability.[4] To escape the membranes, viruses exploit the changing chemical environment of the E-L compartments. The progressively reducing conditions in the maturing endosomes trigger the viral-coating spike polypeptide unfolding and the exposure of hydrophobic protein residues that penetrate in the E-L membrane, forming pores, and allowing viral release into the cytosol.

Nanobiotechnology can imitate these sophisticated mechanisms to improve nano-carrier E-L translocation into the cytoplasm. In terms of particle design, these steps implicate: (i) surface receptor binding triggering endocytic cell entry, (ii) protection of the cargo/particle, and finally, and (iii) E-L membrane permeabilization and escape, preserving cell viability.

E-L membrane translocation of nanomaterials can be improved changing the shape/surface properties of the nanostructures.[5,6] Some nanomaterials, such as CNTs, display extraordinary invasive properties, penetrating through many different biological membranes.[7–13] Moreover, after protein-functionalization CNTs behave as smart nanomaterials interacting with cell surface receptors activating endocytosis,[9–11,14] being transitorily confined inside endosomes,[9] and eventually translocating into the cytosolic compartment.[13,15,16]

Here we test a new hypothesis for the design of a coating system that mimics a viral particle. This synthetic covering allows a controlled, reproducible and viable nano/micrometric particle cytoplasmic invasion using CNTs. Figure 1 depicts the fabrication process of these “viral-mimetic particles” (VMPs) that consist of central spherical fluorescently labelled silica particle cores (*f*-SiO_2) of ~500 nm, externally coated with partially desorbed CNTs (ca. 500–1000 nm) that imitate the viral spike morphology. The fluorescence of the *f*-SiO_2 particles -intact after CNT deposition (Figure S1)- served for intracellular fluorescent particle tracking. As control we used *f*-SiO_2 particles with no CNTs. Similar particles have been reported to undergo receptor-mediated endocytosis and E-L trafficking followed by exocytosis.[17] Finally, to prompt receptor-mediated endocytosis particles were functionalized with culture medium containing serum proteins as previously described.[18]

To investigate the interaction of the VMPs with the cell surface and the entry mechanisms we employed two complementary imaging techniques, (i) phase-contrast life cell imaging, and (ii) scanning electron microscopy (SEM). These studies demonstrated VMPs in close contact with the cytoplasmic projections of the cells being actively transported towards the cell body (Figure 2a, Video S1). This movement, known as “surfing” and typical of viral particles, uses the underlying actin cytoskeleton of the cellular cortex to transport viruses towards the cell body prior to endocytosis.[19]

SEM imaging confirmed the morphological resemblance of the VMPs to many viruses and how, minutes after particle landing on the cell surface, finger-like membranous protrusions -known as filopodia- trapped and embraced the particles (Figures 2b, 2c, arrows). Uncoated particles, on the other hand, were captured by sheet-like membranous extensions -known as lamellipodia- that formed endocytic “cups”.

[a] N. Iturrioz-Rodríguez, E. González-Lavado, Dr. M.L. Fanarraga, Grupo de Nanomedicina-IDIVAL, University of Cantabria, Facultad de Medicina, Herrera Oria s/n, 39011 Santander, Spain
E-mail: fanarrag@unican.es

[b] E. González-Domínguez, L. Marín-Caba, Dr. B. Vaz, Dr. M. Pérez-Lorenzo, Dr. M. A. Correa-Duarte
Department of Physical Chemistry & Department of Organic Chemistry, Center for Biomedical Research (CINBIO), Southern Galicia Institute of Health Research (IISGS), and CIBERSAM, Universidade de Vigo, 36310 Vigo, Spain.

[#,*] These authors contributed equally to this work.

Supporting information for this article is given via a link at the end of the document.

10.1002/anie.201707769

WILEY-VCH

COMMUNICATION

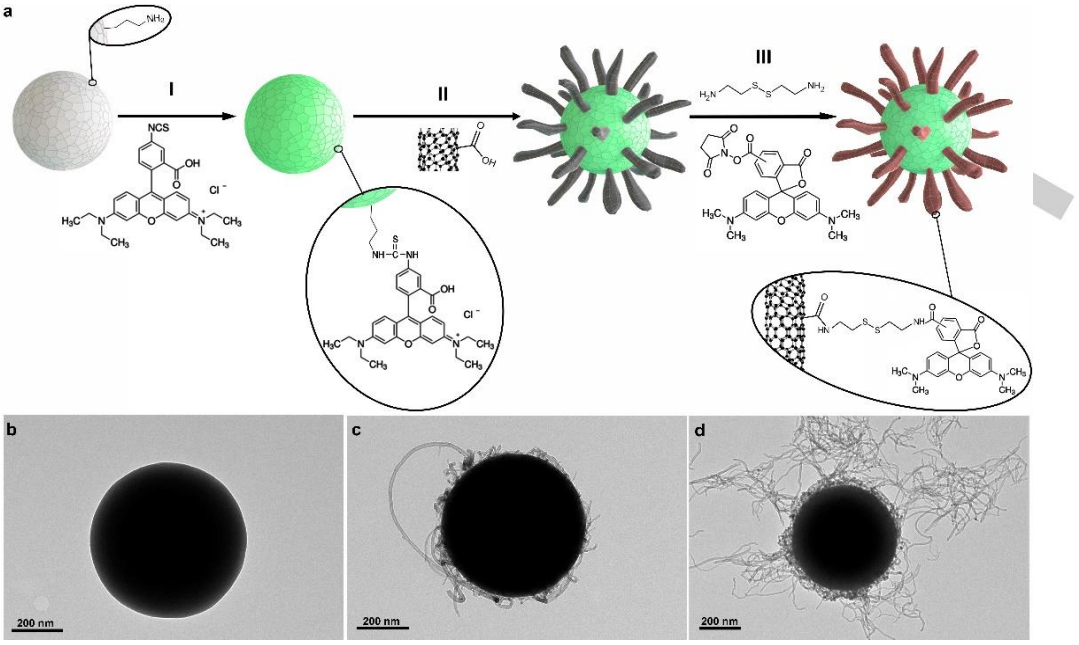


Figure 1. a) Sequential synthetic steps of the VMPs: (I) fluorescent labelling of the SiO_2 uncoated particles (*f*-SiO_2), (II) deposition of CNTs on VMPs, and (III) 5-TAMRA-VMP fluorescent labelling. TEM images of (b) *f*-SiO_2 and *f*-SiO_2 coated with (c) a single, or (d) double CNT coating.

Quantification of particle uptake revealed that, during the first 2 h of exposure, VMPs were internalized by human cells twice as fast as the uncoated *f*-SiO_2 (30% vs 15%, respectively) (Figure 3a). At this point in time, >95% of the cells contained randomly located intracellular particles. Longer exposure times concentrated the VMPs in the centrosomal region of the cells (Figures 3b, S2).

Interestingly, while VMPs were only occasionally observed extracellularly, a relatively constant number of extracellular uncoated *f*-SiO_2 particles were detected suggesting a continuous cycle of particle endocytosis-exocytosis. To test this hypothesis, cells were repeatedly washed to remove extracellular particles. *De novo* particle release was observed minutes after cell wash (Figure 3c, Table S1). This experiment proved that the two types of particles underwent different intracellular destinies, supporting a role of the CNT coating in intracellular particle retention.

To document the intracellular routes and destinies for the two types of particles, we used transmission electron microscopy (TEM) on cytoplasmic cell sections. While intracellular *f*-SiO_2 nude particles were always observed coated with membranes, most VMPs were completely devoid of membranes and fully integrated in the surrounding cytoplasmic environment (Figure S3c). Particle escape to the cytosol was demonstrated using live-cell confocal *time-lapse* microscopy. The process of VMP escape started with the polarization of the particles towards the E-L membrane (Video S2). Particle polarization was concomitant to a progressive E-L contents release indicative of vesicle membrane permeabilization, and preceded particle release to the cytosol (Figure 4a, insets 1-2). Most VMPs were devoid of membranes approximately 72 h after initial cell contact (Figure S4). Interestingly, cell necrosis, apoptosis, or anti-proliferative effects were not detected in cultures days after the treatment with VMPs, thus suggesting the E-L particle escape mechanism was a cell-viable process (Figure S5). TEM imaging served to further confirm vesicle permeabilization by the CNT coating of the VMPs (Figures 4b, S3c). Many VMPs appeared in the cytosol, some presenting shredded membranes intermingling with the CNTs of their coating

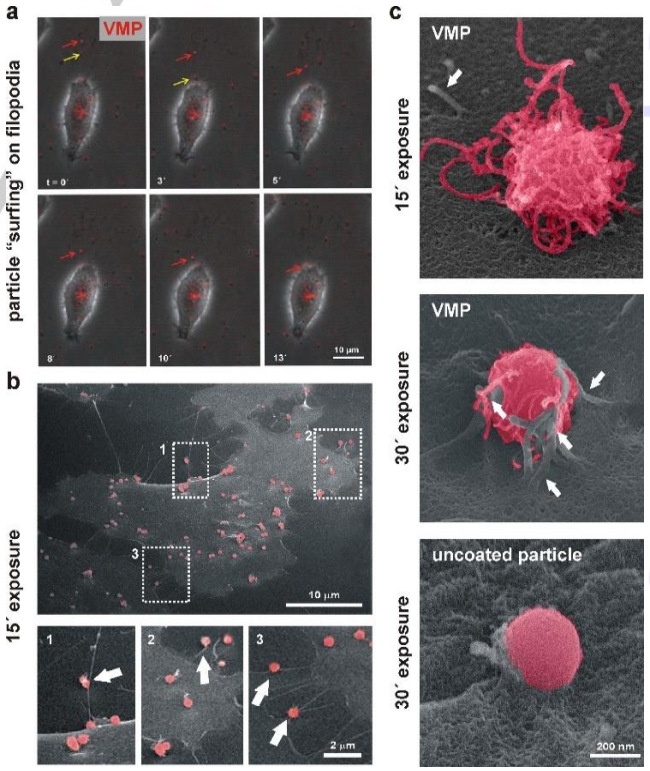


Figure 2. a) VMPs surfing on filopodia (Video S1). Arrows point at VMPs (red fluorescence). b) SEM images of VMPs on human. c) Cell surface contacts with VMPs and *f*-SiO_2. Arrows point at cell filopodia embracing VMPs. Uncoated particles are captured by lamellipodia. Particles are pseudocoloured in pink.

10.1002/anie.201707769

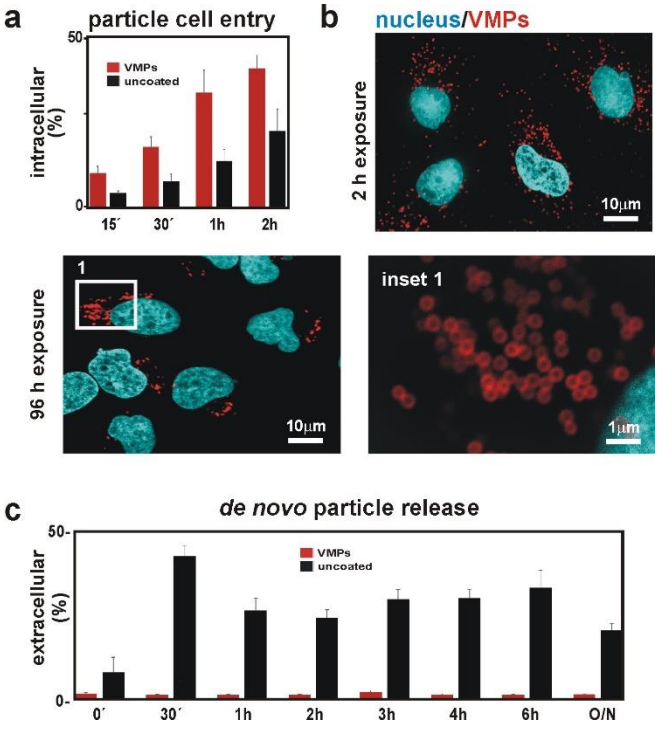


Figure 3. a) Quantification of particle uptake by HeLa cells. All values are significant for a t >5; p<10^{-5}; degrees of freedom DF = 30). b) VMPs appear throughout the cell cytoplasm soon after cell contact becoming perinuclear hours after engulfment (red channel). c) Quantification of *de novo* particle release (after cell wash) (Figure S2) All values are statistically significantly <5 x 10^{-3}; (DF = 30) (Table S1). Total intracellular particles at 12 h = 100%.

All these experiments support the idea that this nanotube coating participates in particle escape to the cytosol (i) penetrating, (ii) piercing, and permeabilizing the E-L membranes, (iii) finally destroying the integrity of this subcellular compartment, (iv) allowing VMP release into the cytosol.

To investigate this hypothesis, we used a covalent cystamine linker to decorate the surface of the CNTs with the fluorescent dye 5-TAMRA (Figure 1-III). This would serve to confirm (i) CNT protein biocorona removal, (ii) CNT-dye unlinking, and finally, (iii) lysosomal membrane permeabilization followed by (iv) dye release into the cytoplasm. As it is typical for CNTs, the fluorescence of this dye is virtually quenched when linked to the surface of the nanotube, becoming detectable after exposure to reducing conditions *in vitro* (Figure S6). The E-L lysosomal reducing conditions *in vivo* [1,2,20] permitted dye fluorescence detection in the cytoplasm of HeLa cells 24 h after 5-TAMRA-CNT endocytosis. Likewise, cells treated with 5-TAMRA-VMPs presented a patent cytoplasmic 568 nm fluorescent emission 24 h after VMP uptake (Figure S7). These results confirm CNT protein striping and E-L membrane permeabilization as part of the mechanism of VMP escape into the cytosol.

As is the case for the viral-surface spike proteins, CNT protein removal in the lysosomes would change the surface properties of these nanofilaments, exposing the apolar/hydrophobic regions of the nanotubes. This modification enhances their affinity for the E-L lipidic membranes, triggering membrane penetration, perforation, permeabilization and ultimately, VMP release into the cytosol. These steps in the VMP escape reproduce the stages of viral translocation into the cytoplasm (Figure 5). Lipid tail protrusions have also been shown to mediate the insertion of gold nanoparticles into model cell membranes.[21]

Summarizing, here we describe the internalization and escape route of a new synthetic particle-coating as a model for the fabrication of future nanostructured materials destined for different applications in the cytosolic or nuclear realms. The design of the CNT coating allows mimicking the viral cellular uptake as well as the exceptional ability of these microorganisms to escape from the E-L into the cytosol without affecting the viability of the host cells. Given that, this strategy of cytoplasm particle invasion based on artificial nano-coatings is applicable to a wide range of morphologies from the nanometer to the micrometer scale, including hollow, porous and solid geometries. Thus, this design constitutes an extraordinary tool for the modelling of therapeutic architectures. In line with this, it is of special relevance the administration of therapies that involve biodegradable biomolecules such as proteins and/or nucleic acids Furthermore, these structures allow endless design modifications, for instance, incorporating materials endowed with magnetic, optical or catalytic functionalities, which can expand the applications of these VMPs beyond drug delivery.

In addition, the significantly enhanced surface provided by the CNT-coating -that can be bioconjugated with many different ligands-, together with the cytosolic scape mechanisms serve to drive these VMPs to target cells, providing a unique methodology for the study of many still unexplored intracellular processes within the cell. These features, together with the fact that these engineered nanocoatings can be implemented in almost any carrier to enable E-L escape represent a great added value as a technological development in nanobiotechnology.

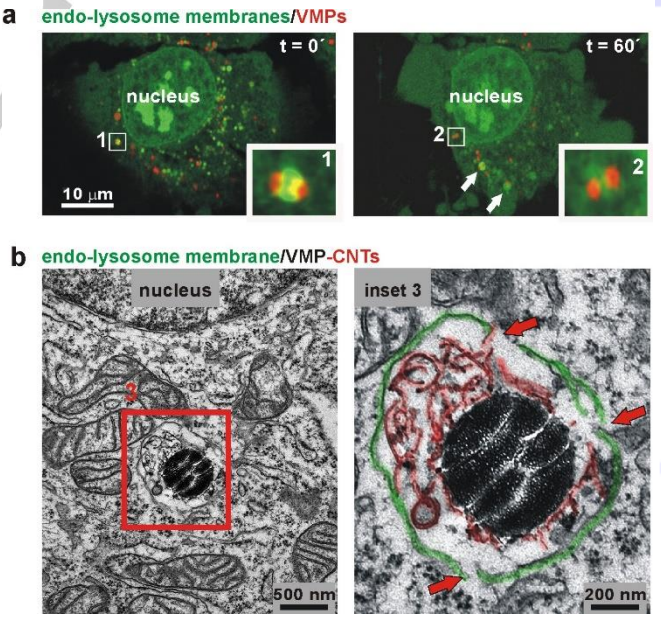


Figure 4. a) Photograms of video S2 demonstrating VMP escape from the E-L membranes. Insets show two VMPs (red channel) inside an E-L and the release of the endosomal contents prior particle escape. b) TEM images of a section of a HeLa cell displaying healthy mitochondria surrounding a permeabilized E-L containing a VMP. The CNTs of the VMP coating (pseudo-coloured in red) are observed in close contact, or piercing, the E-L membrane (green) (red arrows).

10.1002/anie.201707769

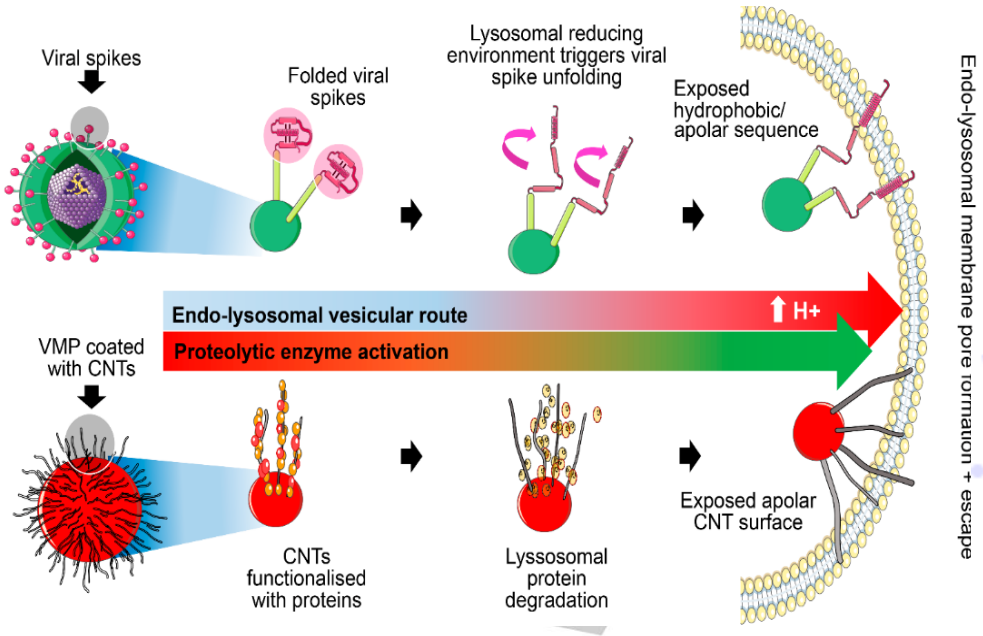


Figure 5. Similarities between viral (green) and VMPs (red) escape mechanisms. (Top) The changing E-L acidic and reducing environment[1,2,20] triggers hydrogen bond release, viral spike protein unfolding, and exposure of hydrophobic protein domains (pink) that penetrate the membranes of the E-L forming small pores that allow the controlled lysosomal contents release, and finally, viral escape into the cytosol; (Bottom) Proteins decorating the VMP CNT biocorona undergo proteolysis in lysosomes[22] exposing the stripped CNT surface that interacts with the E-L membranes, forming small pores that eventually release the particles into the cytosol.[1,2,20]

Acknowledgements

We are grateful to IDIVAL Institute and to L. García-Hevia, JC. Villegas and I. Casafont for technical assistance with microscopy. This work has been supported by the Spanish MINECO Project Refs. PI13/01074, PI16/00496 and CTM2014-58481-R, IDIVAL INNVAL15/15, Xunta de Galicia (Centro Singular de Investigación de Galicia-Accreditation 2016-2019 and EM2014/035, European Regional Development Fund (ERDF), Fundación Tatiana Pérez de Guzmán el Bueno.

Keywords: particle release, CNT, endocytosis, lysosomal permeabilization, cytoplasmic invasion.

[1] J. M. Kinchen, K. S. Ravichandran, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 781–95.

[2] J. B. Lloyd, *Biochem J.* **1986**, *237*, 271–2.

[3] H. K. Shete, R. H. Prabhu, V. B. Patravale, *J. Nanosci. Nanotechnol.* **2014**, *14*, 460–74.

[4] J. Mercer, M. Schelhaas, A. Helenius, *Annu. Rev. Biochem* **2010**, *79*, 803–833.

[5] N. A. Kotov, *Science.* **2010**, *330*, 188–189.

[6] Z. Chu, S. Zhang, B. Zhang, C. Zhang, C.-Y. Fang, I. Rehor, P. Cigler, H.-C. Chang, G. Lin, R. Liu, et al., *Sci. Rep.* **2014**, *4*, 3050–3061.

[7] Q. Mu, D. L. Broughton, B. Yan, *Nano Lett.* **2009**, *9*, 4370–4375.

[8] A. Albini, V. Mussi, A. Parodi, A. Ventura, E. Principi, S. Tegami, M. Rocchia, E. Francheschi, I. Sogno, R. Cammarota, et al., *Nanomedicine Nanotechnology, Biol. Med.* **2010**, *6*, 277–288.

[9] L. Lacerda, J. Russier, G. Pastorin, M. A. Herrero, E. Venturelli, H. Dumortier, K. T. Al-Jamal, M. Prato, K. Kostarelos, A. Bianco, *Biomaterials* **2012**, *33*, 3334–3343.

[10] P. N. Yaron, B. D. Holt, P. A. Short, M. Lösche, M. F. Islam, K. N. Dahl, *J. Nanobiotechnology* **2011**, *9*, 1–15.

[11] K. Maruyama, H. Haniu, N. Saito, Y. Matsuda, T. Tsukahara, S. Kobayashi, M. Tanaka, K. Aoki, S. Takanashi, M. Okamoto, et al., *Biomed Res. Int.* **2015**, *2015*, 793186.

[12] X. Shi, A. von dem Bussche, R. H. Hurt, A. B. Kane, H. Gao, *Nat. Nanotechnol.* **2011**, *6*, 714–719.

[13] K. Kostarelos, L. Lacerda, G. Pastorin, W. Wu, S. Wieckowski, J. Luangsivilay, S. Godefroy, D. Pantarotto, J.-P. Briand, S. Muller, et al., *Nat. Nanotechnol.* **2007**, *2*, 108–113.

[14] Q. X. Mu, D. L. Broughton, B. Yan, *Nano Lett.* **2009**, *9*, 4370–4375.

[15] L. Rodriguez-Fernandez, R. Valiente, J. Gonzalez, J. C. Villegas, M. L. Fanarraga, *ACS Nano* **2012**, *6*, 6614–6625.

[16] L. Garcia-Hevia, R. Valiente, J. L. Fernandez-Luna, E. Flahaut, L. Rodriguez-Fernandez, J. C. Villegas, J. Gonzalez, M. L. Fanarraga, *Adv. Healthc. Mater.* **2015**, *4*, 1640–1644.

[17] I. I. Slowing, J. L. Vivero-Escoto, Y. Zhao, K. Kandel, C. Peeraphatdit, B. G. Trewyn, V. S. Y. Lin, *Small* **2011**, *7*, 1526–1532.

[18] E. González-Domínguez, N. Iturrioz, E. Padín-González, J. C. Villegas, L. García-Hevia, M. Pérez-Lorenzo, W. J. Parak, M. A. Correa-Duarte, M. L. Fanarraga, *Int. J. Nanomedicine* **2017**, *12*, 6317–6328.

[19] M. J. Lehmann, N. M. Sherer, C. B. Marks, M. Pypaert, W. Mothes, *J. Cell Biol.* **2005**, *170*, 317–325.

[20] B. Arunachalam, U. T. Phan, H. J. Geuze, P. Cresswell, *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 745–750.

[21] R. C. Van Lehn, M. Ricci, P. H. J. Silva, P. Andreozzi, J. Reguera, K. Voïtchovsky, F. Stellacci, A. Alexander-Katz, *Nat. Commun.* **2014**, *5*, 4482.

[22] P. Bohley, P. O. Seglen, *Experientia* **1992**, *48*, 151–157.

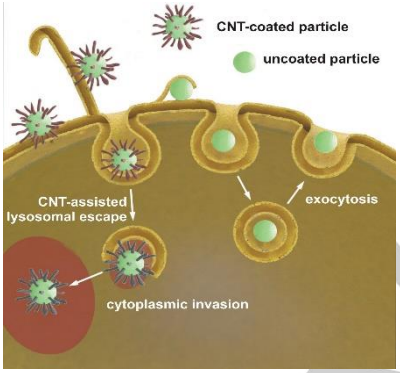
10.1002/anie.201707769

WILEY-VCH

Entry for the Table of Contents

COMMUNICATION

Escaping the lysosomal membranes: An engineered nanocoating composed of CNTs enables a life-compatible nano/micrometric particle invasion into the intracellular realm.



N. Iturrioz-Rodríguez, E. González-Domínguez, E. González-Lavado, L. Marín-Caba, B. Vaz, M. Pérez-Lorenzo, M. A. Correa-Duarte and M. L. Fanarraga

Page No. – Page No.
A synthetic getaway biomimetic strategy for cytoplasm particle invasion