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COMMUNICATION

Delivery of SiC-Based Nanoparticles into Live Cells Driven by Cell-Penetrating Peptides SAP and SAP-E

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Delivery of SiC-based nanoparticles (SiC-NPs) into living eukaryotic cells is facilitated in the presence of cell-penetrating peptides, cationic (SAP) and anionic (SAP-E). The SiC-NPs surface functional group modification combined with rational CPP selection introduces an additional mode of the delivery control.

Nanometer-sized semiconductor particles (NPs)[1-5] arouse considerable interest of researchers in the last decade, first of all due to the promising biomedical applications.[1,2] Their fluorescent properties have already found use in modern imaging technologies,[8-10] providing the opportunity to visualize biological processes by fluorescence labelling at tissue,[11] cellular[12] and single-molecular[13] levels. Comparing to other known fluorophores, NPs have higher quantum yields and molecular extinction coefficients of the fluorescence, narrower emission spectra, size-dependent emission, higher chemical and photo-stability.[14,15]

Main problems to be addressed in the fluorescence labelling using NPs are inefficient penetration of the NPs through cell membranes and their non-specific delivery inside the cells. Many studies were devoted to developing various strategies for NP internalization into the living cells, with the special emphasis on selective targeting of the NPs to different cellular organelles and compartments.[16] Most of the reported delivering strategies were based on conjugation of the NPs to biomolecules,[16,17] which might also drive them to specific cellular locations. The use of cell-penetrating peptides (CPPs) as the carrier molecules is of particular interest in this respect. The CPPs linked to nano-cargoes efficiently promote cargo internalization without significant cytotoxic effects,[18] and, in many cases, were shown to target specific cellular organelles like nuclei or mitochondria.[19] Therefore, the CPPs can not only facilitate the NP uptake by cells, but also deliver them to specific sites inside the cells.[20-22]

Among the numerous NP types reported to date, ZnS-covered CdSe quantum dots (CdSe@ZnS QDs) are probably the most studied. Important advances in their preparation[23-25] and conjugation to different biomolecules, including CPPs,[16] led to spectacular bioimaging applications. However, other NP types which might offer advantages in comparison with the CdSe@ZnS QDs are studied much less; obviously, their potential has to be explored as well. We disclosure in this paper our results of such an exploration into the intra-cellular delivery of NPs prepared by electrochemical anodization of a low resistivity grade (<1 Ω·cm) bulk 3C-SiC polycrystalline wafer (SiC-based NPs, SiC-NPs†).[26] Using this type of the NPs for bioimaging application, one can benefit from simplicity of their preparation procedures, low potential toxicity,[27,28] as well as from their ability to form stable colloidal solutions in water and other polar solvents.

Recently, charge-driven selective localization of fluorescent SiC-NPs in cells was reported.[29] Manipulation of the SiC-NPs surface functional groups was shown to actively govern the intracellular fate of the SiC-NPs. The as-prepared SiC-NPs possessing carboxylic groups on their surface,[30] are deprotonated and charged negatively at physiological pH (SiC-NPs(-), Figure 1). Reaction shown in Figure 1a transformed the carboxylic moieties into positively charged aminoethylcarboxamide functional groups, protonated under the physiological conditions (SiC-NPs(+), Figure 1b).[29] The negatively charged SiC-NPs(-) targeted cell nuclei; under the same conditions, the positively charged SiC-NPs(+) were unable to penetrate inside the nuclei and accumulated in the cytosol. Excellent fluorescence of the SiC-NPs(+) and SiC-NPs(-) (Figure 1c) allowed efficient visualization of the cells treated by the NPs of this type by a fluorescence microscope.
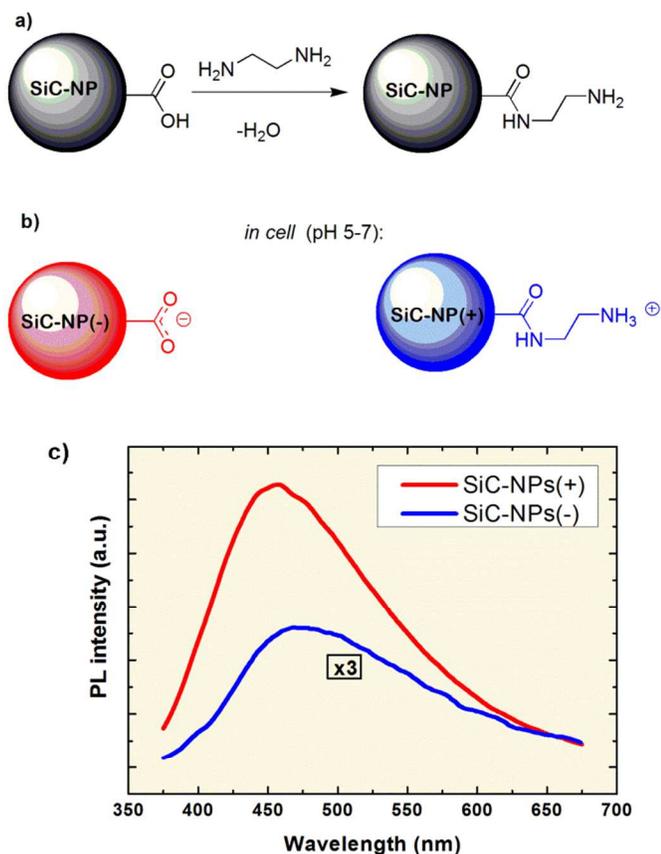
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Figure 1. Chemical modification of the carboxylic groups on the surface of the SiC-NPs (a), their ionization states under cellular internalization conditions (b), and photoluminescence spectra (excitation at 343 nm) of the as-prepared SiC-NPs used in this study (c).

Targeting the cell nuclei by the as-prepared negatively charged SiC-NPs(-) was shown to be closely associated with the process of cell division. In the normal fibroblast or epithelial cells, the overall uptake of SiC-NPs(-) and their accumulation in the nuclei are both markedly reduced when cells are confluent and do not divide with no nucleus targeting at the full confluence state.[31]

In this paper, we report on improved strategy to achieve efficient and selective internalization of the SiC-NPs into live cells, which is based on the CPP-assisted delivery. This strategy can be applied to both normally dividing and confluent cells. For each given surface modification of the SiC-NPs, we propose to choose the corresponding carrier CPP, selecting it from the large known pool,[18] so that the SiC-NP surface functional groups could form non-covalent bonds with the complementary side-chain groups of the CPP and therefore are "matched". In particular, amphiphilic arginine-containing CPP "Sweet Arrow Peptide" (SAP, (Val-Arg-Leu-Pro-Pro-Pro)₃)[32,33] and its analogue, glutamate-containing SAP-E (Val-Glu-Leu-Pro-Pro-Pro)₃)[34] were chosen to be used as the carrier peptides. We choose them because they possess differently charged side chains (positively charged SAP and negatively charged SAP-E); both cationic SAP and anionic SAP-E were artificially designed and reported to efficiently internalize into eukaryotic cells.[31-33] In accordance to our "matching" hypothesis, the positively charged SAP can form non-covalent conjugates with the negatively charged SiC-NPs(-). Coulombic attraction between the charged groups should be reinforced in this case by the hydrogen bonds between the carboxylic and guanidinium moieties (**Figure 2a**), that is important for the CPP to compete efficiently for the binding sites with "protein corona" of the nanoparticles formed in living systems.[35-37] Analogously, the anionic SAP-E can interact with the positively charged SiC-NPs(+), as schematically shown in the **Figure 2b**. Importantly, the SAP and SAP-E peptides were purposely designed as amphipatic PPII helices,[31-33] so their charged functional groups are located at one side of their molecules, positioned to form multiple non-covalent bonds with one nanoparticle.
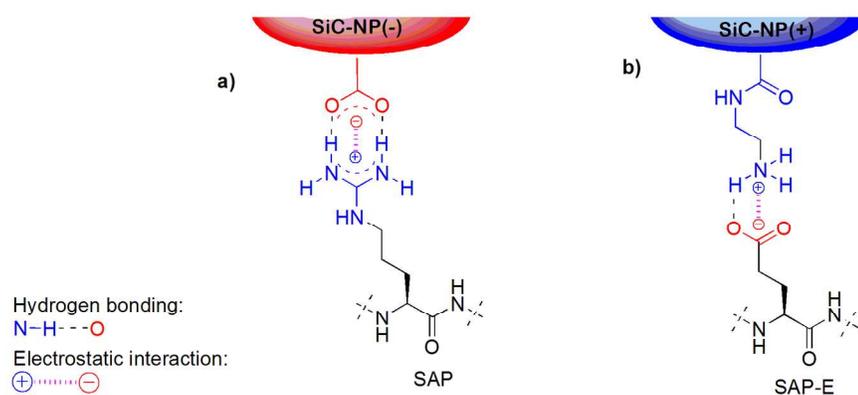

Figure 2. Expected non-covalent interactions between oppositely charged SiC-NPs and CPPs. As-prepared anionic SiC-NP(-) interacting with arginine side chains of SAP (a); and cationic SiC-NP(+) interacting with glutamate residues of SAP-E (b).

Only a small fraction of the peptide might interact reversibly with the NPs surface, due to non-covalent nature of the interaction. Indirect indication of this is the zeta-potential of the nanoparticles, which changed only slightly upon the peptide addition (for example, from +100 to +97 mV in the system

SiC-NPs(+)/SAP-E). However, this might be sufficient to affect the transport of the NPs through the cell membrane.

The experimental procedure for the (SiC-NPs)/(carrier peptide) conjugation, as it relied upon the non-covalent interactions, was very simple. The corresponding nanoparticle solutions were just mixed with the SAP or SAP-E solutions and kept at room

temperature for several hours (see Supporting Information for the details). Cell cultures of 3T3-L1 murine fibroblasts (American Type Culture Collection, Manassas, VA, USA) were exposed to the formed solutions overnight. Fluorescence of the cell cultures incubated with the NP solutions and that of non-treated (control) cells was observed by means of a fluorescence microscope, and increase in the fluorescence over the

background in the presence of the NPs alone or their complexes with the CPPs in different combination was determined. The results are illustrated in Figure 3.
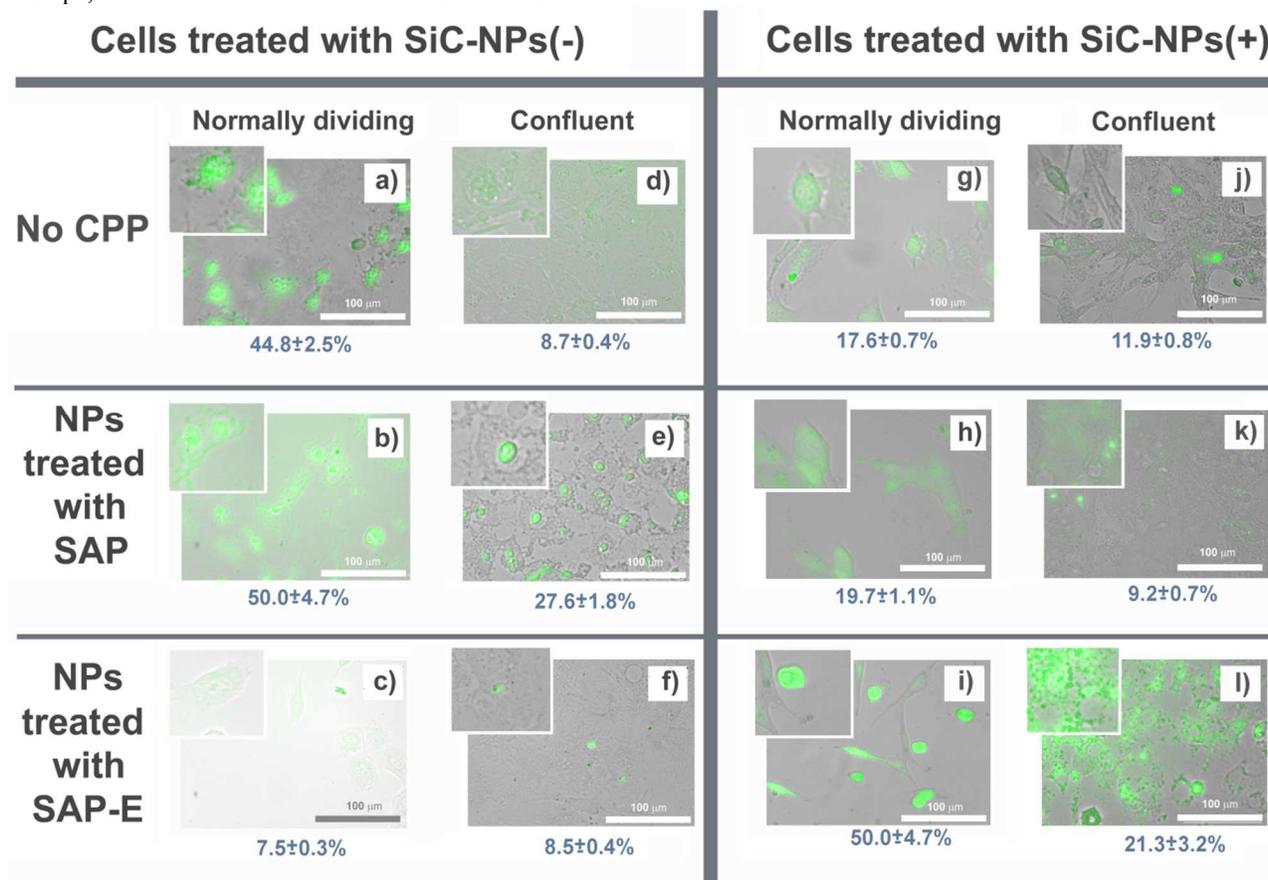

Figure 3. Fluorescent microscope images of the 3T3-L1 cells (normally dividing and in a confluent state) exposed to SiC-NPs(-) alone (a, d); SiC-NPs(+) alone (g, j); treated with SiC-NPs(-)/SAP complex (b, e); treated with SiC-NPs(+)/SAP complex (h, k), treated with SiC-NPs(-)/SAP-E complex (c, f); treated with SiC-NPs(+)/SAP-E complex (i, l). Inserts are parts of the corresponding images magnified by 2. Enhancement of the fluorescence intensity over the background (in percents) is shown under each picture.

As can be seen from Figure 3, treatment of the SiC-NPs by a carrier peptide indeed facilitated the intracellular entry of the nanoparticles, but only for certain SiC-NPs/carrier combinations, in agreement to our "matching" hypothesis. For example, SiC-NPs(-) treated by SAP increased the fluorescence of the cells higher that the SiC-NPs(-) alone, as can be judged from the average fluorescence per cell counting (compare the **Figures 3a** and **3b, 3d** and **3e,** and see the Supporting information). The most pronounced effect of the CPP presence was observed when the cells were at full confluence: the non-proliferating confluent cells were efficiently labelled by the SiC-NPs(-) incubated with SAP (**Figure 3e**), contrary to the as-prepared peptide-free SiC-NPs(-) (**Figure 3d**). So, the presence of SAP conferred to the SiC-NP(-) the ability to enter densely grown non-dividing cells, and increased overall cell entry of the particles. In addition, the CPP-driven delivery of the SiC-NPs(-) into the proliferating living cells was nuclei-selective, as it can be seen in **Figure 3e** (insert). Similar

selectivity was previously observed[29] when the peptide-free SiC-NPs(-) penetrated inside the nuclei of the non-confluent cells (**Figure 3a**).
Enhancement of the intracellular SiC-NPs(+) delivery was observed when the nanoparticles were treated by SAP-E (compare **Figures 3g** and **3i**, **3j** and **3l**). Drastic difference in the imaging ability between the SiC-NPs(+) alone and the SiC-NPs(+)/SAP-E pair was noticed: while the bare SiC-NPs(+) stayed predominantly in the cytosol (**Figure 3g**), the nanoparticles treated with SAP-E accumulated exclusively in the cell nuclei (**Figure 3i**). This difference cannot be explained simply by recharging the SiC-NPs(+) in the presence of the SAP-E: as we noticed above, zeta-potential of the SiC-NPs(+)/SAP-E complexes (+97 mV) was only slightly lower than that of the as-prepared SiC-NPs(+) (+100 mV). Obviously, the CPP-induced NPs intracellular delivery should be explained by a different mechanism than in the case of NPs without SAP.

Most importantly, while the SiC-NPs(-)/SAP complex penetrated efficiently into the confluent cells (see **Figure 3e**, the CPP-cargo pair is "matched"), incubation of the cell cultures close to the confluent state in the presence of the SiC-NPs(+)/SAP complexes did not lead to significant fluorescent labelling (**Figure 3k**), most probably because stable conjugates did not form in this case (the CPP-cargo pair is "mismatched"). Obviously, in order to achieve efficient NPs cell delivery, the choice of the carrier peptide is highly important. It should consider the SiC-NPs functional groups, their ionization state, as well as the nature of the peptide side chains.

It is also important to note that SAP, SAP-E and their complexes with the SiC-NPs in different combinations at the concentration of 0.4 mg/mL had no significant effect on cell proliferation and did not induce cell death. This was confirmed by real-time cell analysis (see the Supporting Information). We also measured gene expression of specific cell markers of stress, cell death, inflammation, etc. In all the cases the transcription of a selection of genes specifically activated by apoptosis, inflammation, mitochondrial stress or unfolded protein response did not significantly modulate in response to SAP or SAP-E at 0.4 mg/mL. This indicated that the cells were perfectly safe under the experimental conditions, and the SAP/SAP-E did not induce significant traumatic shock to the cells.

Conclusions

Cell-penetrating peptides SAP and SAP-E are efficient tools for controlled fluorescent cell labelling by the SiC-NPs. The labelling of the cells is simple and practical, not dependent on the cell state, so it can be recommended for bioimaging applications in live cell cultures. The cationic SAP is most suitable for delivering of the as-prepared SiC-based NPs containing carboxylic groups on their surface. The NH_2-grafted SiC-NPs(+) are best delivered into the cell nuclei by anionic SAP-E. We anticipate that similar studies will find optimal carrier cell-penetrating peptides (the "matched pairs") for other known NP types.

Notes and references

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† Elemental composition of the nanoparticles can be described as $C_{100.0}H_{104.1}F_{19.5}O_{51.0}$ brutto formula.

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