Electronic Supplementary Material (ESI) for RSC Advances.

Supporting information

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

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Preparation of the SiC-derived nanoparticles (SiC-NPs).

SiC-based NPs were formed as describer earlier[S1] by means of electrochemical anodization of a low resistivity grade (<1 Ω·cm) bulk 3C-SiC polycrystalline wafer. The etching process was performed for 3 hours at a current density of 25 mA/cm2 using a 1:1 HF (50%)/ethanol electrolyte. After the etching, a powder mixture composed mainly of carbon fluoroxide (CFO) NPs and 3C-SiC porous nanostructures was formed. The nano-powder was then naturally dried, removed from the SiC wafer, mechanically grinded and then dispersed in a Krebs buffer solution. The formed colloidal suspension was centrifuged at 10000 g for 5 minutes in order to collect only its top part containing very small (<10 nm) and homogeneously dispersed NPs which can be visualized by atomic force microscope (AFM) as shown in Figure S1-a. Size distribution of the obtained SiC-NPs was estimated from dynamic light scattering measurements (see Figure S1-b) and the average NPs size was found to be in the range of 4-6 nm. FTIR spectrum shown as right insert in Figure S1-b gives an idea about the chemical bonds (C-H, C=O and C-O) in the fabricated NPs. In addition, an elemental composition of the nanoparticles can be described as $C_{100.0}H_{104.1}F_{19.5}O_{51.0}$ brutto formula. The main surface chemical groups of the SiC-NPs are suggested to be carboxylic and ester groups. A UV-vis absorption spectrum of a colloidal suspension of the SiC-NPs in water is described in[S2]. Photoluminescence spectra of the SiC-NPs are found to be centered at 550 nm under excitation at 400 nm.

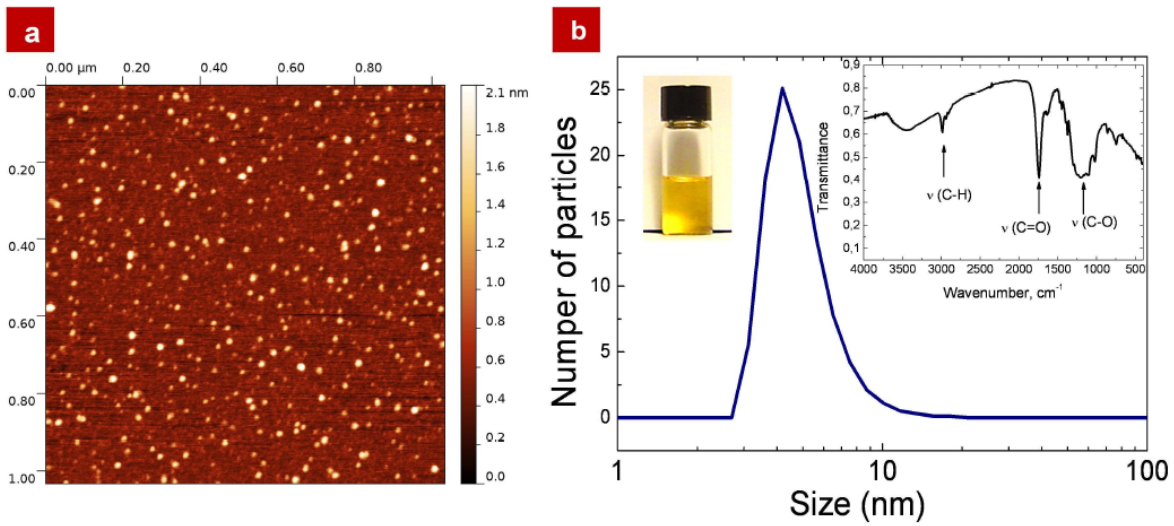

Figure S1: a) AFM image of the used SiC-NPs; b) size distribution of the SiC-NPs, left insert shows colloidal aqueous solution with the dispersed SiC-NPs and right insert gives an idea on their dominating chemical bonds.

Grafting of amino groups on the SiC-NPs surface was performed via the reaction of their surface carboxylic acid groups with ethylenediamine (En).[S3] Initial porous SiC-based nanopowder (10 mg) was refluxed for one hour in a mixture of ethylenediamine with anhydrous *o*-xylene (0.4 mL of En and 1.6 mL of *o*-xylene). Then the liquids were slowly distilled off (in the stream of N_2, the bath temperature did not exceed 160 °C), afterwards

approx. 1 mL of *o*-xylene was added and distilled off to remove the residues of En and H_2O from the reaction mixture. Brown waxy residue was re-dispersed in diluted water HCl solution under sonication and extracted several times by *n*-butanol to remove the excess of *o*-xylene and other possible organic admixtures. Then the excess of Na_2CO_3 solution was added to the remaining acidic solution, the SiC-NPs(+) were extracted in *n*-BuOH and large SiC crystallites were removed by centrifugation at 10 000 g for 5 minutes. Afterwards the SiC-NPs(+) were re-extracted into 0.01 M HCl, the obtained solution was diluted by Dulbecco's Modified Eagle's Medium **(**DMEM).
According to zeta-potential measurements (pH 7), the as-prepared SiC-NPs(-) demonstrated sufficiently high negative zeta-potential close to –30 mV, which was supposedly caused by dissociation of the surface acidic -COOH and possibly Si-OH groups. Protonation of the grafted amino groups in aqueous solution resulted in the positive surface charges. The sample of aminated SiC-NPs(+) synthesized in large excess of En demonstrated high (+100 mV) zeta potential, because according to the FT-IR data, most of the acidic groups in this sample were consumed in the reaction with ethylenediamine, giving a surface covered mainly by NH_2 groups.

Synthesis of the cell-penetrating peptides.

The peptides SAP ($(VRLPPP)_3$) and SAP-E ($(VELPPP)_3$) were obtained using automatic peptide synthesizer (Protein Technologies PS3) by the standard solid-phase peptide synthesis Fmoc protocols.[S4] Proline pre-loaded 2-chlorotrityl resin (85 mg, 100-200 mesh, 0.59 mmol/g loading) was used. Corresponding protected amino acids (6 eq. calculating on the resin loading, 0.3 mmol each: Fmoc-Pro, 101.5 mg; Fmoc-Leu, 106.3 mg; Fmoc-(Pbf)Arg, 195.2 mg; Fmoc-Val, 102.1 mg, and Fmoc-(*t*-Bu)Glu, 127.6 mg) were pre-mixed with PyBOP (benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate, 6 eq.); activation was effected by dissolving this mixture in ethyl diisopropylamine (DIPEA, 8 eq)-DMF (5 mL) immediately before coupling and then added to the resin. A solution of piperidine (20% in DMF) was used for the Fmoc-deprotection (2×15 min). Cleavage and final deprotection was done in a TFA/TIS cleavage cocktail (95:5 v/v) at ambient temperature (3 h). The volatile products were removed *in vacuo*; the residues were purified by reversed-phase high-performance liquid chromatography (RP-HPLC, C18 bonded phase) and then lyophilized.
Chromatographic characterization was performed on a Jasco HPLC system (Japan) using a diode array detector operating at 220 nm. Runs were carried out on a PolyEncap A 300 column (250 mm × 4.0 mm; Bischoff Analysentechnik, Germany). The sample concentration was 1 mg of peptide/mL in eluent A. The mobile phase A was 0.1% trifluoroacetic acid (TFA) in H_2O, and phase B was 0.1% TFA in 80% MeCN – 20% H_2O (v/v).

Treatment of the SiC-NPs with the cell-penetrating peptides SAP and SAP-E.
SiC-NPs(-) and SiC-NPs(+) (4 mg/mL in water) were mixed with SAP (4 mg/mL in water) and SAP-E (4 mg/mL in water) respectively, left standing for 3 hours before exposition to cell cultures.

Study of the SiC-NPs delivery into the cells.
The highly fluorescent suspensions of negatively charged SiC-NPs(-), positively charged SiC-NPs(+), negatively charged SiC-NPs(-) incubated with SAP and positively charged SiC-NPs(+) incubated with SAP-E were added to cell cultures of 3T3-L1 murine fibroblasts (American Type Culture Collection, Manassas, VA, USA). The cells were grown in DMEM supplemented with 10% newborn calf serum, 4 mM glutamine, 4 nM insulin (Actrapid Human; Novo), 10 mM Hepes, 25 μg sodium ascorbate, 100 IU penicillin, 100 μg streptomycin, and 0.25 μg amphotericin B per mL, at 37°C in a water saturated atmosphere

with 5% CO_2 in air, in a Heraeus incubator (BB16). The cells were incubated for 3 hours in the presence of the corresponding nanoparticle solutions diluted by half with the Krebs bicarbonate buffer (pH=7.4). After the incubation period (3 hours) and before their observation, the cells were rinsed twice by the Krebs buffer to eliminate the NPs from their external environment. The fluorescence of the cell cultures incubated with NPs and of control cells was observed by means of a fluorescence microscope (Leica DMI 4000B) with the following filter combination: UV/violet excitation (2.92-3.5 eV) with an observation spectral range <2.64 eV; acquisition time was 1 s; magnification x100. Average fluorescence per cell counting was done using the ImageJ software, and gave the following results: Enhancement of the fluorescense intensity over the background is the following (%): 44.8±2.5 (SiC-NPs(-) alone, normally dividing cells); 50.0±4.7 (SiC-NPs(-) treated with SAP, normally dividing cells); 7.5±0.3 (SiC-NPs(-) treated with SAP-E, normally dividing cells); 8.7±0.4 (SiC-NPs(-) alone, confluent cells); 27.6±1.8 (SiC-NPs(-) treated with SAP, confluent cells); 8.5±0.4 (SiC-NPs(-) treated with SAP-E, confluent cells); 17.6±0.7 (SiC-NPs(+) alone, normally dividing cells); 19.7±1.1 (SiC-NPs(+) treated with SAP, normally dividing cells); 50.0±4.7 (SiC-NPs(+) treated with SAP-E, normally dividing cells); 11.9±0.8 (SiC-NPs(+) alone, confluent cells); 9.2±0.7 (SiC-NPs(+) treated with SAP, confluent cells); 21.3±3.2 (SiC-NPs(+) treated with SAP-E, confluent cells).

Real-time cell analysis.

3T3-L1 cells were seeded at high density 20 000 cells/well for confluence study or at low density 2500 cells/well for proliferation assays in either E-plates 96. Cells were grown at 37°C in 5% CO_2 in DMEM 10% FCS with high glucose, and removed using trypsin 0.05% (PAA Laboratories, Les Mureaux, France). Cell proliferation and/or survival was monitored with the xCELLigence Real-time Cell Analyser (RTCA) System (ACEA Biosciences, Inc., San Diego, USA), which allows label-free monitoring changes of cell number, viability, morphology and quality of cell attachment by measurement of cell to electrode responses of cells seeded in E96-well plates manufactured with integrated microelectronic sensor arrays. RTCA system measures cell surface occupancy, i.e. cell index, taking into account cell number, cell size, and adhesion force. The results are represented as cell indexes (representative results are shown in Figure S2). The results showed that SAP, SAP-E and their complexes with the SiC-NPs in different combinations at the concentration 0.4 mg/mL have no significant effect on cell proliferation or on the cell death.

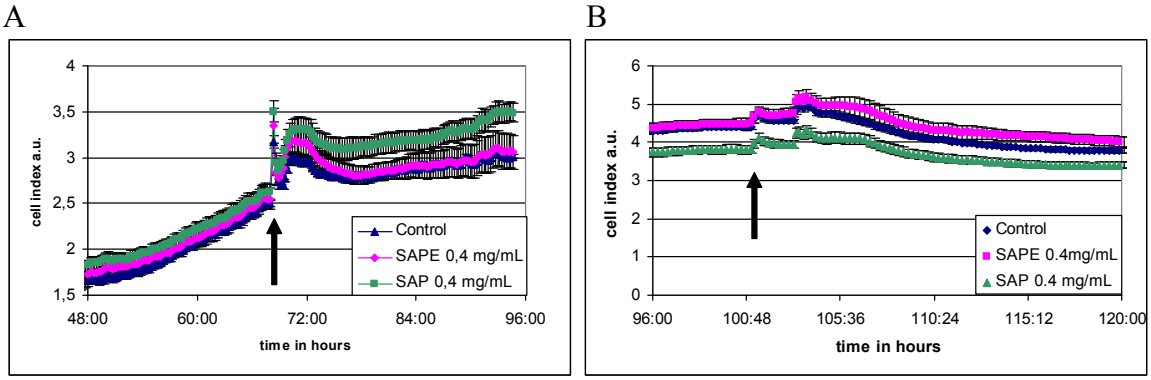

Figure S2: SAP or SAP-E were added to proliferating 3T3-L1 cells (arrow) (A). No significant effect of SAP or SAPE can be seen on cell index of the proliferating cells. 3T3-L1 cells were seeded at high density to reach confluence (B). Addition of SAP or SAPE at 0.4 mg/mL is shown by the arrow. No significant effect of SAP or SAPE can be seen on the cell index.

Gene expression measurements.

Total RNA purifications from 3T3L1 cells were performed using Trizol reagent (Sigma Aldrich, Saint Quentin Fallavier, France). RNA integrity was assessed with the Agilent 2100 Bioanalyzer and RNA 6000 LabChip Kit (Agilent Technologies, Massy, France). First strand cDNAs were synthesized from 1,5 µg of total RNA in the presence of 100 U of Superscript (Invitrogen-Life Technologies, Eragny, France) and random hexamers and oligodT primers (Promega). Real-time quantitative PCR (RT-qPCR) was performed using ABsolute™ QPCR SYBR® Green ROX Mix (Abgene, Courtaboeuf, France) using the primers (Sigma Aldricht) listed in Table S1 with a Nano LC Light Cycler (Roche Diagnosis). Levels of target mRNAs were normalized to hypoxanthine phosphoribosyltransferase 1 expression measured in triplicates and data are presented as mean ± SD. Significativity was measured using Student's test.
Gene transcription was analyzed on 3T3-L1 treated during 24 hr with either 0.4 mg/mL of SAP or SAPE. Both did not significantly modulate the transcription of a selection of genes specifically activated by either apoptosis, inflammation, mitochondrial stress or unfolded protein response (Table S1).

Table S1. Gene transcription analyses on 3T3L1 fibroblasts.

	Control	SAP	SAP-E
COX2	6,3 ± 0,3	4,8 ± 0,8	4,3 ± 0,03
CytB	1735 ± 59	2129 ± 101	1467 ± 9
FAS	3,0 ± 0,6	3,1 ± 0,1	2,6 ± 0,0
GRP78	0,35 0,07	0,30 0,01	0,35 0,01
IL6	3,9 ± 0,8	2,9 ± 0,1	2,3 ± 0,0
TP53	10,3 ± 0,3	8,3 ± 0,4	8,4 ± 0,1
XBP1	0,16 ± 0,01	0,16 ± 0,02	0,17 ± 0,00

References

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[S2] Botsoa, J.; Lysenko, V.; Géloën, A.; Marty, O.; Bluet, J. M.; Guillot, G. *Appl. Phys. Lett.* **2008**, *92*, 173902.
[S3] Serdiuk, T.; Alekseev, S. A.; Lysenko, V.; Skryshevsky, V. A.; Géloën, A. *Nanotechnology* **2012**, *23*, doi:10.1088/0957-4484/23/31/315101.
[S4] Goodman, M.; Felix, A.; Moroder, L.; Toniolo, C., Eds. *Methods of Organic Chemistry (Houben-Weyl). Additional and Supplementary Volumes to the 4th Edition. Vol. 22b-d. Synthesis of Peptides and Peptidomimetics*; Georg Thieme Verlag: Stuttgart/New-York, **2004**.