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Silica nanoparticles as sources of silicic acid

favoring wound healing *in vitro*

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Graphical abstract
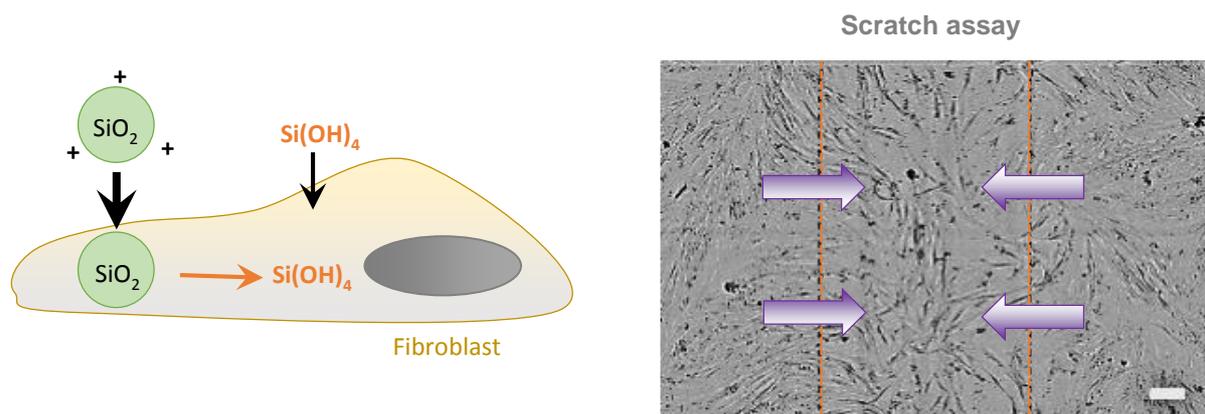
Positively-charged silica nanoparticles are readily internalized by human skin fibroblasts, releasing silicic acid intracellularly, and favoring their proliferation/migration in an *in vitro* wound healing model

1 Highlights

2 • Positively charged SiO2 nanoparticles and soluble silicic acid [Si(OH)4] promote
3   wound healing in vitro
4 • Positively charged SiO2 nanoparticles are more effective than Si(OH)4 in promoting
5   cell migration
6 • Positively charged SiO2 nanoparticles are internalized more readily than Si(OH)4 and
7   dissolve intracellularly releasing Si(OH)4.
8 • SiO2 nanoparticles may be used for the intracellular delivery of bioactive Si(OH)4
9

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18 **ABSTRACT** There is good evidence that certain silicon-containing materials promote would

19 healing and their common feature is the delivery of orthosilicic acid (Si(OH)4) either directly

20 or following metabolism. In this respect, amorphous silica nanoparticles (NP), which dissolve

21 in aqueous environments releasing up to 2 mM orthosilicic acid, may be appropriate 'slow

22 release' vehicles for bioactive silicon. Here we studied the impact of silica NP suspensions

23 (primary particles ~ 10 nm) in undersaturated conditions (below 2 mM Si) with differing

24 degrees of surface charge and dissolution rate on human dermal fibroblasts (CCD-25SK cells)

25 viability, proliferation and migration in a cellular wound model. Silica was shown to be non-

26 toxic for all forms and concentrations tested and whilst the anticipated stimulatory effect of

1 orthosilicic acid was observed, the silica NPs also stimulated fibroblast proliferation and

2 migration. In particular, the amine-functionalized particles promoted wound closure more

3 rapidly than soluble orthosilicic acid alone. We suggest that this effect is related to easy cellular

4 internalization of these particles followed by their intracellular dissolution releasing silicic acid

5 at a faster rate than its direct uptake from the medium. Our findings indicate that amorphous

6 silica-based NPs may favour the delivery and release of bioactive silicic acid to cells,

7 promoting wound healing.

8 **Keywords**: silica nanoparticles; wound healing; silicic acid; fibroblasts

9

1 Introduction

2 Silicon (Si) is the third most abundant trace element in the human body after iron and zinc [1],

3 although its mean daily intake by adults in the Western world is greater than for these latter

4 two elements and estimated to be 20-50 mg [2]. However, unlike zinc and iron, it is not

5 established whether Si has an essential biological role in humans or other mammals. The

6 highest levels of tissue Si are associated with the mammalian connective tissues, such as bone,

7 blood vessels and skin [3-5] and Si depletion of the diet was originally reported to result in

8 stunted growth and malformation of the connective tissues [6,7]. These original findings have

9 not been replicated [8] but more recent studies in humans have reported that Si

10 supplementation, or higher intakes of dietary Si, are associated with an improvement in bone

11 and skin health [9-11]. Mechanisms of action have not been established, although metabolic

12 and/or structural roles in collagen synthesis and stabilization have been proposed [12].

13 Si-based treatments and devices have been reported to aid wound healing and prevent

14 scarring [13,14]. These devices are typically silicone-based and in the form of gels/sheets

15 [15,16]. They are proposed to act as barriers, maintaining the wound moist (*i.e.* hydrated) and

16 protecting it against infection. In addition, it has been suggested that Si is released from the

17 dressing, a fact that can be paralleled with existing evidence in humans that organo-silicons

18 can be metabolized to orthosilicic acid [17]. It is proposed that Si reaches the basal epidermal

19 and dermal fibroblast cells, up-regulating the expression of basic fibroblast growth factor (β-

20 FGF) [18]. Consistent with these observations, inorganic Si-containing materials, such as

21 bioactive glass ointment and silica gel fiber fleeces [19,20], have also been tested for wound

22 healing applications and shown to result in a faster and more efficient cicatrization.

23 Orthosilicic acid ($Si(OH)_4$) is a small molecule that readily diffuses, so maintaining its

24 presence in the wound environment requires some kind of slow release mechanism. In this

25 respect, amorphous silica nanoparticles (NP) may be especially promising candidates for

4

wound healing applications, since not only will they deliver orthosilicic acid up to its solubility limit (*circa* 2 mM) as they dissolve [21], but they can also be loaded with additional therapeutic molecules [22-24]. Although such silica-based drug nanocarriers have been shown to favor cicatrization [25,26], native (i.e. non-functionalized) forms have been less studied for their wound healing potential [27], and, to our knowledge, not at all as 'small' nanoparticles (as opposed to large/aggregated particles) where dissolution to orthosilicic acid is most favorable.

In this context, we have herein investigated here the effects of silica in different forms, soluble (*i.e.* orthosilicic acid) and small nanoparticulate forms (NP, 10 nm in diameter), on the migration and proliferation of human skin fibroblast cells (CCD-25SK), two of the main processes required for cicatrization [28], with the aim of determining if small (i.e. 10 nm) silica NPs *per se* can be useful for wound healing applications.

Materials and methods

Synthesis and characterization of the amorphous silica nanoparticles

Ludox SM30 was purchased from Sigma-Aldrich Chemical Co (Gillingham, UK). Silica NP (referred to as SiNP+10 or SiNP-10 depending on the sign of their Zeta potential) were synthesized according to the literature [29]. Briefly, tetraethyl orthosilicate (TEOS 98 wt%, Sigma-Aldrich Chemical Co) was added to a stirred solution of L-(+)-lysine monohydrate (Sigma-Aldrich Chemical Co) in ultrapure (UHP) water at 60°C. After 72 h at this temperature, SiNP-10 negatively-charged particles were obtained. Some of those particles were post-functionalized with aminopropyl triethoxysilane as described by Badley *et al.* [30]. The obtained positively-charged SiNP+10 were then purified by dialysis (Spectra/Por, 25 mm dialysis tubing with pore diameter of 4.2–5.0 nm, from Carl Roth) with 200 mL particle suspensions being dialyzed five times under magnetic stirring against 2 L of ultrapure water (Milli-Q) for a minimum time of 4 h. Silica NPs were also prepared from a concentrated (~ 7

1 M Si) basic (~ pH 14) sodium silicate solution (Sigma-Aldrich Chemical Co). Following

2 dilution to 10 mM in UHP water, the sodium silicate solution was neutralized with 5 M HCl

3 and aged for 24 h before being used [31]. Particles formed with this method are referred to as

4 NaSi10. For comparison, a soluble orthosilicic acid (Si(OH)$_4$) solution was prepared by

5 dilution of the concentrated sodium silicate solution to 2 mM and neutralization with HCl.

6   The different silica NPs were characterized prior to their supplementation in the cell culture

7 medium. Dynamic Light Scattering (DLS) was used to determine the hydrodynamic diameter

8 of the silica NPs in UHP water. The reading (three measurements of at least 10 readings) was

9 carried out at an angle of 175˚ to the incident beam (632.8 nm). Zeta potential was measured

10 on a Malvern Zetasizer (NanoZS) with the particles dispersed in 10 mM KCl.

11   In addition to size and surface charge determination, the dissolution of the silica NPs in cell

12 culture medium was determined by measuring, over time, the increase in concentration of

13 soluble silicic acid after separation from the non-soluble fraction by ultrafiltration (Nanosep 3

14 kD ultrafilter units; Pall). The ultrafiltrates were digested in sodium hydroxide and analyzed

15 by ICP-OES (JY Horiba Ultima 2C; Lonjumeau, France) as previously described [32], with Si-

16 measurement at 251.6 nm. The rate of dissolution (R) of the silica NPs was obtained from

17 fitting the equation below [21] to the data (**eq.1**)

18 $$C = C_{eq}(1 - 10^{-RSt\,/\,C_{eq}})$$ (1)

19   where C is the apparent Si concentration at time t and C_{eq} is the apparent Si concentration at

20 equilibrium, as obtained from ICP-OES measurements.

21

22 **Cell culture**

23   Human skin fibroblasts, CCD-25SK were obtained from ATCC (Manassas, USA) as a frozen

24 aliquot. Cells were resuscitated in complete growth medium [Minimum Essential Medium

25 (MEM), supplemented with 5% foetal calf serum (FCS; Sigma-Aldrich Co., UK), 100 µg mL^{-1}

1   penicillin and streptomycin (Invitrogen Ltd, Life Technologies UK) and 0.25 μg mL^{-1}

2   fungizone (Invitrogen Ltd, Life Technologies UK)] and grown in Nunc T75 culture flasks (75

3   cm^2; VWR International, UK) at 37 °C in a humidified atmosphere containing 5% CO_2. At

4   approximately 80% confluence, cells were detached from the cell culture flasks with 0.1%

5   trypsin/0.02% EDTA solution, resuspended in complete culture medium (*i.e.* supplemented

6   MEM medium) and were either passaged or used in the experiments described below. Cells

7   were used between passage 10-20 for the experiments described herein.

8   Silicon supplemented media were prepared fresh by diluting the appropriate amounts of the

9   silica stocks (nanoparticle suspensions or 2 mM soluble orthosilicic acid solution) in complete

10   growth medium to obtain final Si concentrations between 0.01 mM and 0.5 mM Si.

11

12   **Cell viability assays**

13   Cell viability and proliferation of CCD-25SK cells, following incubation with the Si

14   supplemented media, was quantitatively evaluated with the fluorescent and non-toxic cell dye

15   Resazurin (Invitrogen, Life Technologies UK). Briefly, the Si supplemented media and

16   controls were removed after 24 and 72 h incubation and the cells rinsed with phenol red–free

17   MEM. Cells were then incubated with phenol red-free MEM containing 10% (44 μM)

18   Resazurin for 6 h at 37°C in a humidified atmosphere containing 5% CO_2. Aliquots of the

19   media were then transferred to a flat-bottom 96-well plate and the absorbance measured at 540

20   nm and 595 nm on an optical plate-reader (Labsystems Multiskan RC, Thermo Scientific Inc,

21   USA). To check for false positive results originating from the silica NPs, a control containing

22   just particles was incubated with the Resazurin dye, and this gave negligible absorbance values.

23

24   **Cell proliferation assay**

7

1   CCD-25SK cells were seeded in Nunc 48-well plates at an initial cell density of 5,000 cells per

2   well with 1 mL of complete growth medium and left to adhere overnight. The medium was

3   then replaced with the freshly prepared Si-supplemented media. Complete growth medium

4   without Si supplementation acted as the control, whilst complete growth medium supplemented

5   with 50 μM ascorbate-2-phosphate (A-2-P), a promoter of cell proliferation, or 250 nM

6   dexamethasone, a promoter of cell differentiation, were used as positive and negative controls,

7   respectively. Phase-contrast images were acquired every 2 h, over a 24 to 72 h period, with the

8   Essen IncuCyte Zoom live cell imaging system (Essen BioScience Inc., USA). In some

9   experiments cells were exposed to the Si-supplemented media for just 6 h followed by

10   incubation in complete growth medium (*i.e.* without Si supplementation) for the remaining 66

11   h. The acquired phase contrast images were analysed using the IncuCyte software to determine,

12   at each time point, percent cell confluence (*i.e.* % of the well area occupied by cells). The

13   analysis procedure used by the IncuCyte software was defined using control cells at different

14   stages of confluence. Each Si concentration was examined in at least three independent

15   experiments with six replicates per treatment per experiment.

16

17   *In vitro* **wound healing assay**

18   CCD-25SK cells were seeded in 96-well image-lock plates (Essen BioScience Inc), at a

19   density of 8,000 cells per well in complete growth medium and grown to confluence.

20   Thereafter, a wound (scratch) was created in each well using a 'Wound Maker' tool (Essen

21   BioScience Inc). This specialized, high precision tool creates a similar size wound in each well

22   of the plate. The wound was then rinsed thoroughly with complete growth medium to remove

23   the detached cells and fresh Si-supplemented medium or controls were added. Phase-contrast

24   images were then acquired every 2 h, over a 96 h period, with the Essen IncuCyte Zoom live

25   cell imaging system. Phase contrast images were analyzed using the IncuCyte Zoom software,

1 first to determine the initial wound area and then to determine the percentage change (increase)

2 in cell confluence within the wounded area at each time point. Each treatment was assessed in

3 at least two independent experiments, with six replicates per treatment per experiment.

4

5 **Statistical analysis**.

6 Results are shown as means ± SD, unless otherwise stated. The Shapiro–Wilk Normality Test

7 used to check the data was normally distributed. Differences between control and treatments

8 (silica treatments, A-2-P and dexamethasone) were evaluated by Student t-test. A value of P <

9 0.05 was considered significant. To correct for multiple testing, a Bonferroni correction was

10 applied to the P values (i.e. P/number comparisons).

11

12

13 **Results and Discussion**

14 **Characterization and dissolution behavior of the silica nanoparticles**.

15 The different silica NPs were characterized prior to their use in the cellular studies (**Table 1**).

16 All four types of silica NPs investigated (SiNP+10, SiNP-10, Ludox SM30 and NaSi10) had

17 an initial diameter (D) around 10 nm (in UHP water) but DLS studies indicated that SiNP+10

18 and SiNP-10 tended to rapidly form aggregates of 100 nm and 400 nm in diameter,

19 respectively, in the cell culture medium (**Table 1**).

20

21

22

23

24 **Table 1.** Characterization of the silica nanoparticles used herein

Silica NPs	D	ζ	dm/dt	dm/dt_{cells}	NP + 6 h	NP + 72 h

	$(nm)^a$	$(mV)^b$	$(mM.h^{-1})^c$	$(mM.h^{-1})^c$	$(\%)^d$	$(\%)^d$
Ludox SM30	11	-30 ± 5	1.18 ± 0.1	0.87 ± 0.2	90	30
SiNP-10	$10 (100)^e$	-22 ± 4	0.70 ± 0.08	0.43 ± 0.02	95	30
SiNP+10	$10 (400)^e$	$+23 \pm 4$	0.55 ± 0.03	0.54 ± 0.06	95	30
NaSi10	10	n.d.	2.45 ± 0.3	5.85 ± 0.5	10	< 1

1   [a] diameter, in UHP water, obtained by dynamic light scattering. [b] zeta potential in UHP water
2   ($\pm$SD). [c] Dissolution rates (dm/dt) were determined in cell culture medium with and without
3   cells (mean $\pm$ SE). [d] NP recovery after filtration of a 50 μM Si suspension incubated for 6 h and
4   72 h in the presence of cells. [e] Size of aggregates in cell culture medium are reported in
5   parentheses. n.d. = not determined.

6

7   The dissolution of the different silica NPs in the cell culture medium at 37˚C and 5% CO_2

8 was studied by monitoring the concentration of silicic acid by ICP-OES following

9 ultrafiltration of the culture media through 3 kD membrane (**Figure 1**). Calculated dissolution

10 rate (dm/dt) was the highest for NaSi10 and the lowest for SiNP+10 whereas Ludox SM30 and

11 SiNP-10 showed similar intermediate values. The presence of the CCD-25SK cells in the

12 culture medium impacted particle dissolution but not in a consistent manner for all particle

13 types (**Table 1**). Overall, after 6 h incubation in the cell culture medium, at 37˚C and 5% CO_2,

14 and in the presence of the fibroblast cells, Ludox SM30, SiNP-10 and SiNP+10 remained

15 mostly intact, whereas the NaSi10 particles dissolved readily (90% dissolution). After 72 h,

16 about 70 % of the former particles were solubilized whereas the latter were fully dissolved.
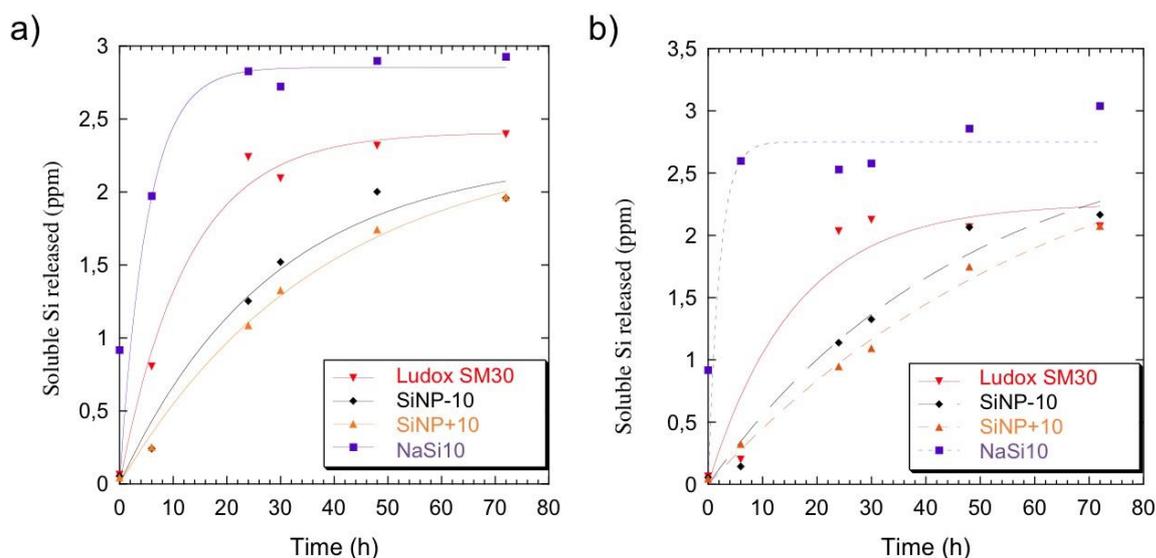
Figure 1. Dissolution kinetics of the different <mark>silica</mark> nanoparticles in complete growth medium at 37°C and 5% CO_2 in the presence (b) and absence (a) of human skin fibroblast cells (CCD-25SK). The experimental data were fitted with curves corresponding to equation 1 (see main text) to determine the dissolution rates of the different silica nanoparticles

The dissolution rate of pure silica NPs under standardized aqueous conditions depends on two main parameters: their specific surface area and the condensation degree of the silica network. Since the particles studied here are non-porous and of similar diameter, the kinetics of soluble silicic acid release can be related to the connectivity of the Si-O-Si network. In this respect, the higher dissolution rate of NaSi10 particles is consistent with the fact that it is prepared from sodium silicate, resulting in a more 'ionic' type network than SiNP10 that are synthesized from silicon alkoxides [33]. Moreover, it has been shown that silica NPs modified with silane moieties (*i.e*. SiNP+10 particles) dissolve less readily than 'bare' silica NP because their surface is less accessible to water molecules [34]. Furthermore, the positive surface charge of SiNP+10 particles favors the deposition of a protein corona [35], that may also impact on dissolution kinetics of the particles. All these considerations explain why SiNP+10 particles have the lowest dissolution rate amongst all silica NPs tested.

1   It is interesting to note that the presence of cells did appear to influence silica NP dissolution

2   (**Table 1**), albeit not in any apparent consistent direction. Dissolution rate might be decreased

3   due to either adsorption of particles on the cell membrane or internalization of the particles,

4   especially in the case of SiNP+10 whose positive charge was shown to favor their cellular

5   uptake [36]. In contrast, the presence of cells accelerated the dissolution of NaSi10. Since these

6   particles are rapidly converted into a soluble form in the acellular media, it is possible to

7   suggest that, for this system, a 'consumption' of silicic acid, being related to its adsorption or

8   internalization, shifts the equilibrium towards more dissolution. Altogether, these results

9   indicate that the silica NP solutions are dynamic systems where colloidal species coexist with

10   soluble silicic acid and whose behavior and thus cellular effects are directed by key physico-

11   chemical parameters, namely size, silica condensation degree and surface charge.

12

13   **Effect of silica nanoparticles on viability and proliferation of human dermal fibroblasts**

14   **Figure 2** shows the metabolic activity, i.e. viability and proliferation as obtained from the

15   Resazurin test, of human skin fibroblasts (CCD-25SK) exposed to the different silica

16   treatments at 50 and 100 µM for 24 h and 72 h. No significant effect of silica addition on

17   metabolic activity was observed after 24 h of contact. However, at 72 h, cells incubated with

18   soluble silicic acid at both concentrations, or with SiNP+10 at 100 µM, showed a significant

19   ($P \leq 0.04$) increase in metabolic activity (by 13-18 %). At higher concentrations of up to 500

20   µM Si, there was no difference between any of the treatments and controls (**Figure S1**),

21   consistent with prior findings for soluble silicic acid in different cell types [37,38]. At 72 h,

22   metabolic activity of the cells was also increased (by ~ 10%) with the positive control, A-2-P,

23   and decreased (by ~ 10%) with the negative control, dexamethasone (**Figure 2**).

1
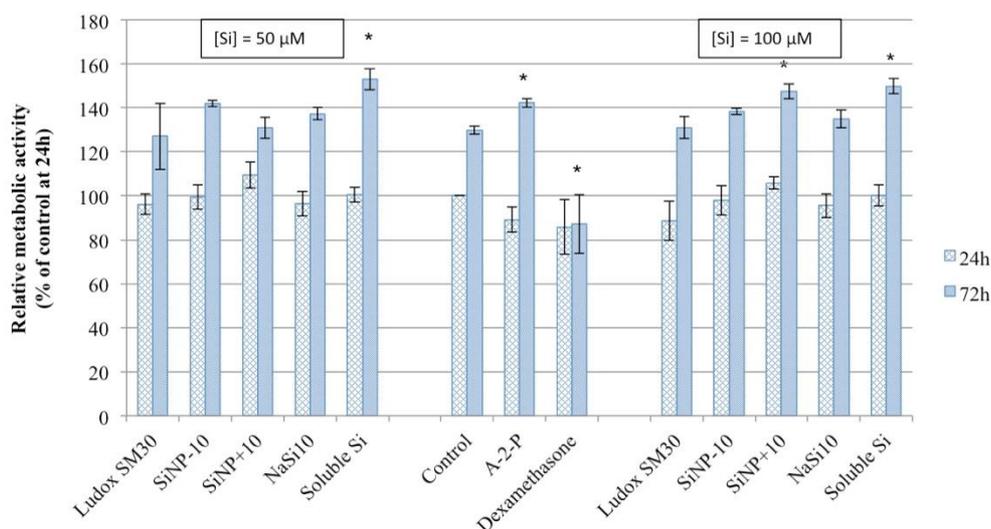
Figure 2. Metabolic activity (i.e. cell viability and proliferation) of human skin fibroblasts
(CCD-25SK cells) exposed to silica (50 and 100 μM Si) from different sources for 24 and 72
h. Ascorbate-2-phosphate (A-2-P; 50 μM) and dexamethasone (250 nM) were used as positive
and negative controls, respectively. Data are mean ± SD of 2 independent experiments with 6-
replicates per treatment per experiment.

Therefore, no detrimental effect of the silica NPs or soluble silicic acid on the metabolic
activity of the cells was observed, regardless of dose or incubation time. This may appear to
contradict previous reported findings for silica NPs with similar size [39-42]. However the
concentrations used here (*i.e.* 50-100 μmol Si/L) are well-below those reported to decrease the
viability of human skin cell lines ($\geq$ 360 μmol Si/L) [42]. In addition cytoxicity is indeed cell
line/cell type specific [43]. We doubt whether aggregation of the particles in the medium
explains their lack of toxicity, as at least Ludox SM30 and NaSi10 particles remained well-
dispersed in the cell culture medium (**Table 1**).

Considering cell proliferation, as monitored by live cell imaging, both SiNP-10 and Ludox
SM30 induced small (7-18%) but significant increases in confluence above the untreated
control after 48 and 72 h incubation (P $\leq$ 0.03) and were comparable to the positive control A-

13

1   2-P (increase of 15-19%) (**Figure 3**). NaSi10 also significantly increased cell growth but at

2   higher concentrations (> 200 μM Si; **Figure S2**). However, the most marked increase in

3   confluence (by 31-59%) was observed with soluble orthosilicic acid. Results were similar for

4   the two Si concentrations (50 and 100 μM) investigated. SiNP+10 had almost no impact on

5   cell proliferation. Dexamethasone, the negative control, decreased the growth of the cultured

6   cells by ~ 70%.

7   The addition of soluble silicic acid up to 30 μM Si was previously shown to enhance both

8   the metabolic activity and proliferation of bone cells [44]. A similar effect is observed here

9   that does not vary significantly with Si concentrations of 50 μM or 100 μM. For silica NPs, the

10   cellular effects appear to be strongly dependent on the surface charge and the dissolution

11   behavior of the particles. The negatively-charged particles had only minor effects on the cells:

12   the SiNP-10 and Ludox SM30 particles, that exhibit low rates of dissolution, slightly favored

13   metabolic activity and cell proliferation while NaSi10 particles, despite their higher rate of

14   dissolution, had a significantly lower impact on cell behavior at comparable concentrations.

15   The positively-charged SiNP+10 particles, with a slower dissolution rate, increased metabolic

16   activity similarly to soluble silica and were less efficient in promoting proliferation. This

17   suggests that both colloidal and soluble forms of silica can impact on the cellular behavior but

18   in a different manner.

(a) 50 µM Si

(b) 100 µM Si
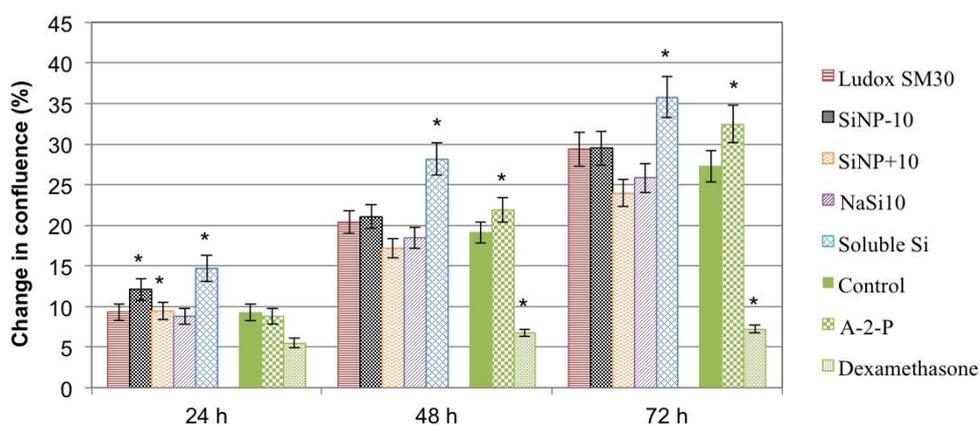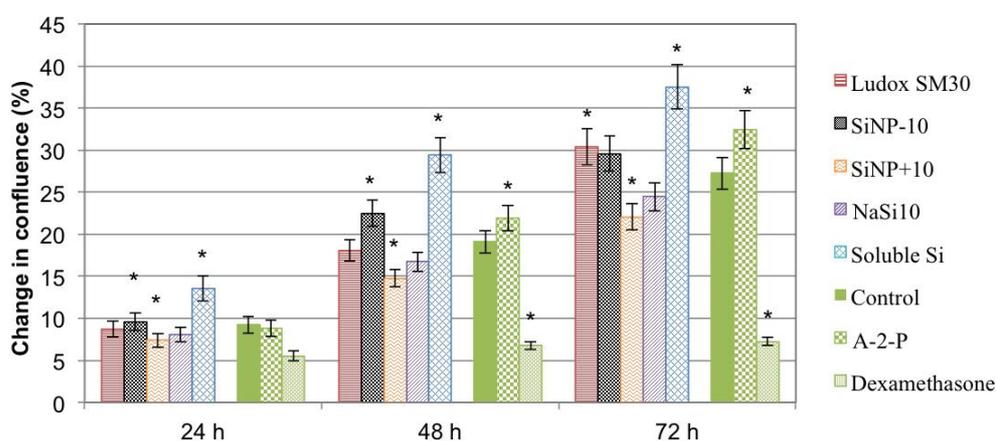
Figure 3. Change in cell confluence of human skin fibroblasts (CCD-25SK cells) exposed to silica at (a) 50 µM and (b) 100 µM Si from different sources for 24-72 h. Ascorbate-2-phosphate (A-2-P; 50 µM) and dexamethasone (250 nM) were used as positive and negative controls, respectively. Data are mean ± SD of 3 independent experiments with 6-replicates per treatment per experiment.*, $p < 0.05$ as compared to control cells maintained in the absence of additional Si

Effect of silica nanoparticles on skin fibroblasts in a wound healing model

The effects of soluble silicic acid and silica NPs on skin fibroblast cells were investigated in a wound closure model (termed 'scratch assay'). Overall, the silica-treated cells migrated and proliferated to cover the wounded area faster than untreated control cells (**Figure 4**).

15

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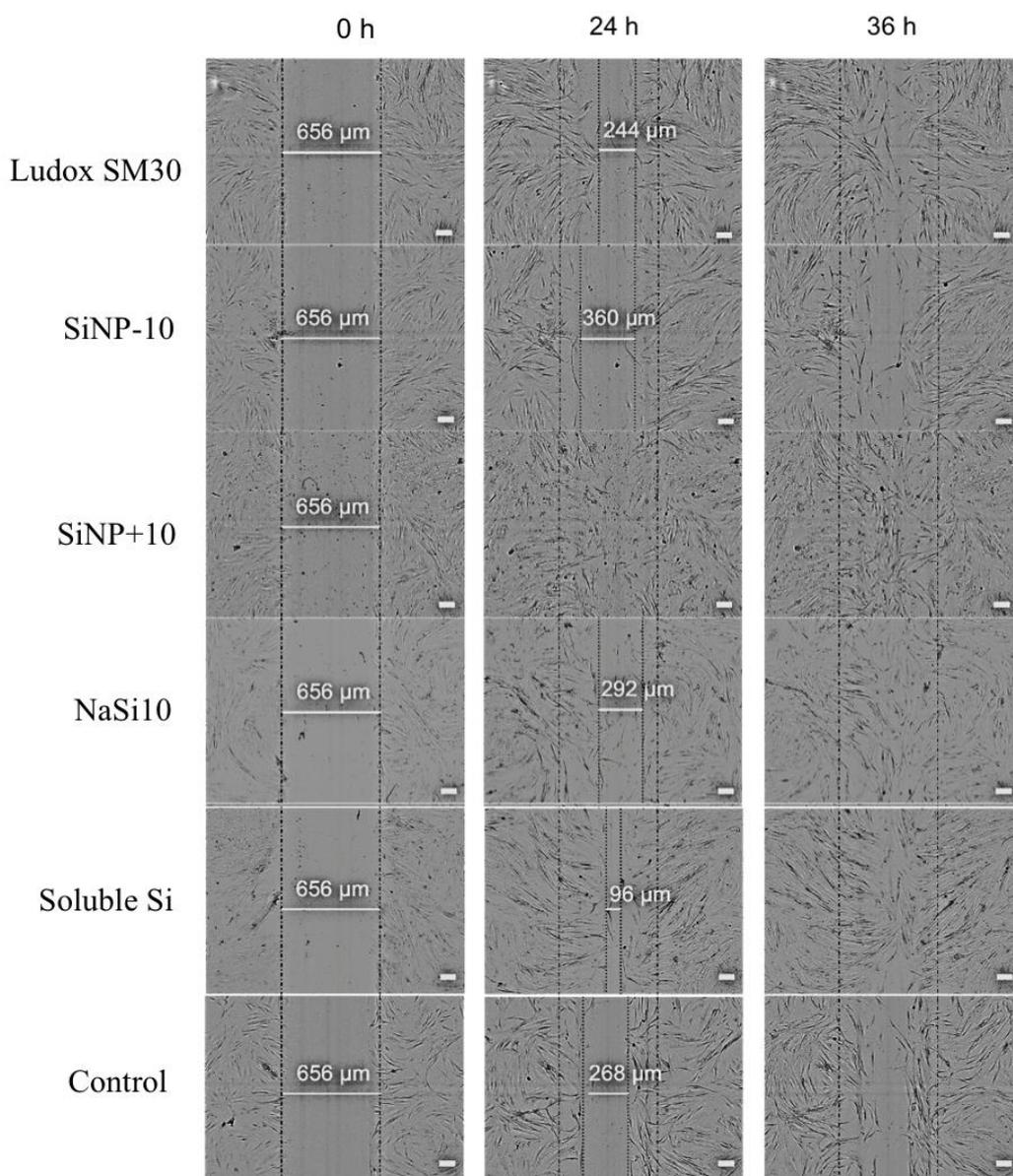
3   **Figure 4**. Phase contrast images of human skin fibroblast (CCD-25SK) monolayers after

4   creation of the wound in the wound closure assay and following 24 and 36 h exposure to the

5   silica treatments at 50 μM Si. Scale bar = 100 μm

6

7   However, not all silica treatments were as effective in promoting wound closure (**Figure 4** and

8   **Table 2**). Soluble silicic acid and SiNP+10 were the most efficient in promoting wound

9   closure. Interestingly the SiNP+10 achieved an apparently greater effect than soluble silicic

1 acid, strengthening the previous assumption that the particles *per se* had a specific effect on

2 wound closure above being just a source of silicic acid in the medium.

3

4 **Table 2.** Time required to attain cell confluence in the wound area for the different treatments

	Time to attain cell confluence (h)	
Treatments	50 µM Si	100 µM Si
Control	42.8 ±13.6	42.8 ±13.6
Soluble silicic acid	31.0 ± 5.4	38.7 ± 1.2
NaSi10	40.6 ± 13.3	39.8 ± 18.1
SiNP-10	38.9 ± 9.8	37.8 ± 3.6
SiNP+10	19.0 ± 4.8	nd
Ludox SM30	38.9 ± 7.5	39.0 ± 6.4

5 Time constant in hours, obtained by fitting the exponential rise observed on the graph
6 corresponding to the increase in confluence in the wound area, nd = not determined.

7

8 In order to clarify this point, human skin fibroblasts were exposed to the silica treatments for

9 only 6 h of incubation to limit NP dissolution in the cell culture medium. Again, there was no

10 detrimental effect of the silica NPs on cell growth. Noticeably, SiNP+10 induced the greatest

11 increase in cell proliferation at all the time points investigated (**Figure 5**). In contrast, the effect

12 of soluble silicic acid was muted implying that its influence on cell activity requires longer

13 exposure times (*e.g.* 24-72 h as in Figure 2). Therefore silica NPs appear to have two effects in

14 the wound healing model: (1) a rapid, direct stimulatory effect and (2) a delayed effect due to

15 soluble orthosilicic acid being released from the dissolution of silica NP in the culture medium.

16

(a) 50 µM Si, 6 h of exposure
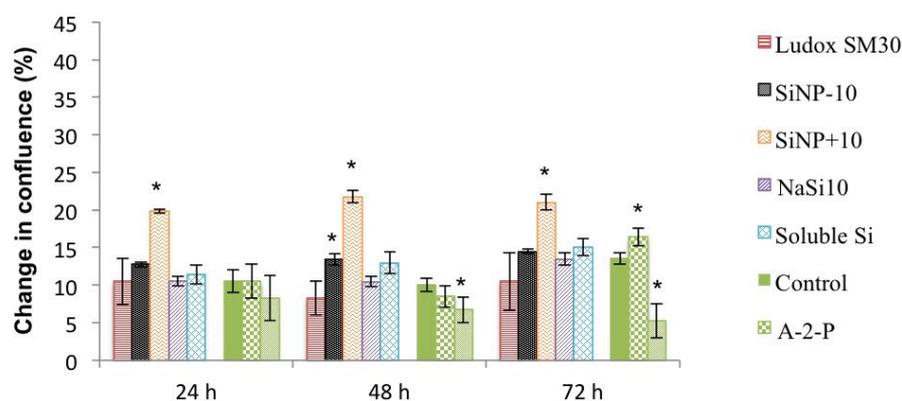
(b) 100 µM Si, 6 h of exposure

1
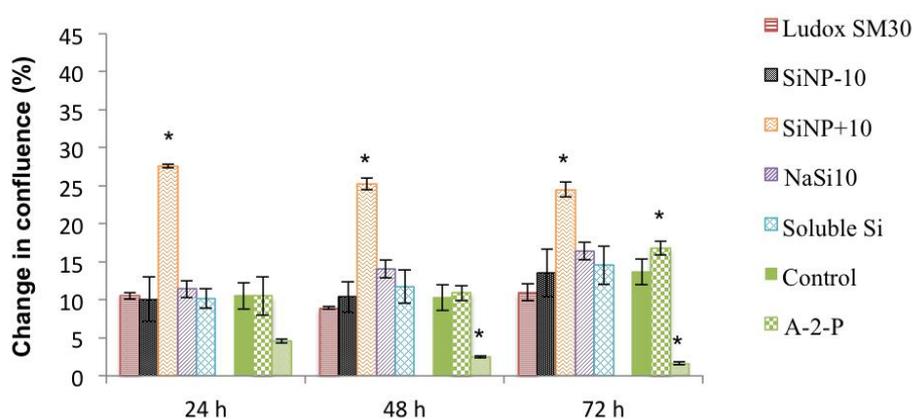
Figure 5. Change in cell confluence of human skin fibroblasts (CCD-25SK cells) exposed to silica at (a) 50 and (b) 100 µM Si from different sources for just 6 h. After 6 h, media was removed and fresh basal media (without added Si) was added for the remaining incubation period (18-66 h). Ascorbate-2-phosphate (A-2-P; 50 µM) and dexamethasone (250 nM) were used as positive and negative controls. Data are mean ± SD of 3 independent experiments with 6-replicates per treatment per experiment.*, $p < 0.05$ as compared to control cells maintained in the absence of additional Si

9

In parallel, analysis of the incubation medium for total Si, before and after 6 h exposure to cells, showed that for Ludox SM30, NaSi10 and SiNP-10, almost all the added Si was recovered in the medium at 6 h (**Figure 6**). In contrast, only 20-25% of the supplemented

1   SiNP+10 was recovered at 6 h and an additional 50-70% was found in the fresh medium at the

2   end of the 76 h incubation period. Therefore these particles appear to be retained, either by

3   adsorption or internalization, by the cells in the first hours of the experiments and then released

4   over time. Fluorescence microscopy imaging of the cells confirmed that SiNP+10 were readily

5   taken up by the fibroblast cells while negatively-charged SiNP-10 appeared to adhere to the

6   cell surface (**Figure S3**). Although 3D confocal imaging, which allows full interpretation of

7   events in the Z plane, would be necessary to fully confirm these data, they are consistent with

8   previous findings [45-47].   These results are also in agreement with the previously-

9   demonstrated intracellular dissolution of positively-charged silica nanoparticles, followed by

10 the release of soluble species from the cells [36].

11
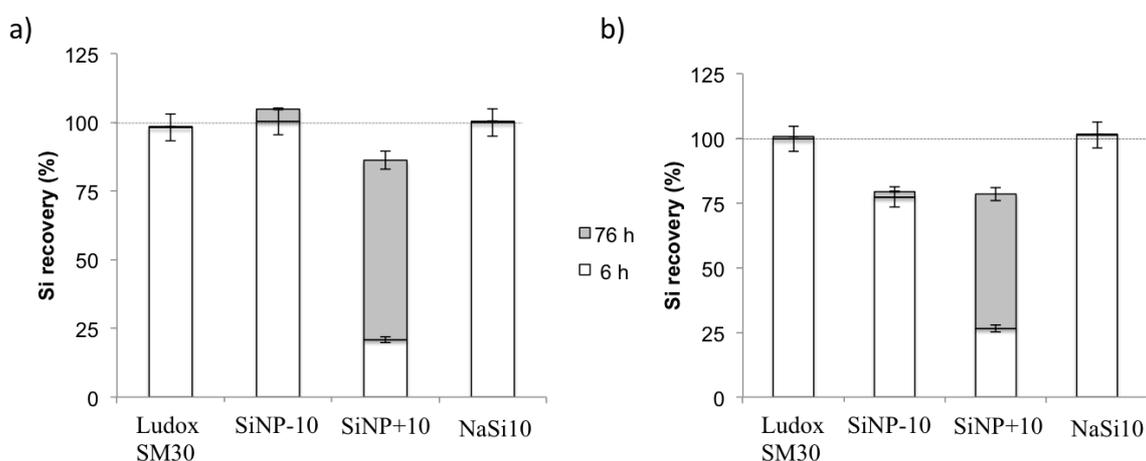
12

13 **Figure 6.** Silicon recovered in the incubation medium after exposure of human skin fibroblasts

14 (CCD-25SK cells) to silica from different sources at (a) 50 and (b) 100 µM Si. Cells were

15 exposed to the silica treatments for 6 h exposure, after which the medium was removed and

16 fresh medium (without added Si) was added for the remaining 70 h. Data are mean ± SD of 2

17 independent experiments with 3-replicates per treatment per experiment.

18

1   Taken together, these data suggest that soluble silicic acid is the bioactive form of silica but

2   is internalized slowly so that a delay is required before its effect on cell behavior can be

3   observed. In contrast, positively-charged SiNP+10 are rapidly taken-up by the cells, can

4   undergo intracellular dissolution and would thus release silicic acid both inside and outside the

5   cells. Finally, the internalization of negatively-charged particles appears limited, in good

6   agreement with the literature [48]. Hence it is very likely that their positive effect on

7   proliferation and migration is due to the silicic acid released by dissolution in the medium

8   which is taken up by the cells. Indeed our experiments also indicate that adsorption of silicic

9   acid and silica particles on the cell surface can impact on the solubility equilibria and thus on

10   the availability of the different silica forms. Therefore, in the absence of detailed information

11   on the full endocytosis/exocytosis processes associated with silica nanoparticles and on the

12   resulting silica speciation, a precise correlation between the physico-chemical characteristics

13   of the silica NP and their observed biological effects remains difficult. These findings

14   exemplify the complexity of the aqueous chemistry of silica [49], and especially the co-

15   existence of soluble and condensed forms, their relative amounts being highly dependent on

16   [Si], pH, ionic strength and interactions with surrounding ions, molecules or surfaces [22, 47,

17   50-52]. This is one plausible reason for the ongoing lack of understanding of the mechanisms

18   by which silica influences biological processes, despite multiple observations showing that it

19   does happen.

20

21   **CONCLUSION**

22   Despite all of the work on the toxicity of nanosilica, our results show that ultrafine silica

23   nanoparticles, at appropriate concentrations, can *positively* impact the proliferation and

24   migration of CCD-25SK human skin fibroblast cells in a way that is anticipated to promote

25   wound healing. Whilst supporting the previous assumption that silicic acid is the bioactive

1   form of silica, our findings also suggest that this form is not readily internalized by the cells.

2   However, provided that they exhibit a positively-charged surface a rapid cellular uptake of

3   small silica NP can occur. We propose that they thus act as a bolus delivery form of bioactive

4   silicic acid that becomes available upon intracellular dissolution. Hence, in parallel with a

5   continued effort to analyze the influence of soluble silica on cell metabolism, further

6   understanding of the intracellular fate of silica NPs is necessary. At the same time, these results

7   open new perspectives for the application of silica nanoparticles as topical therapy where the

8   intrinsic bioactivity of silicic acid may be combined with that of a loaded drug.

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1 **Supplementary Information**

2 Metabolic activity for Si sources up to 500 μM; change in cell confluence of human skin

3 fibroblasts (CCD-25SK cells) exposed for 24-72 h to NaSi10 ; fluorescence microscopy

4 images of internalization of silica nanoparticles following 24 h exposure of human skin

5 fibroblasts SiNP+10 and SiNP-10 at 100 μM Si.

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12 **Author Contributions**

13 The manuscript was written through contributions of all authors. All authors have given

14 approval to the final version of the manuscript. All authors contributed equally.

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