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Stable DNA

Protection and Deprotection of DNA—High-Temperature Stability of Nucleic Acid Barcodes for Polymer Labeling**

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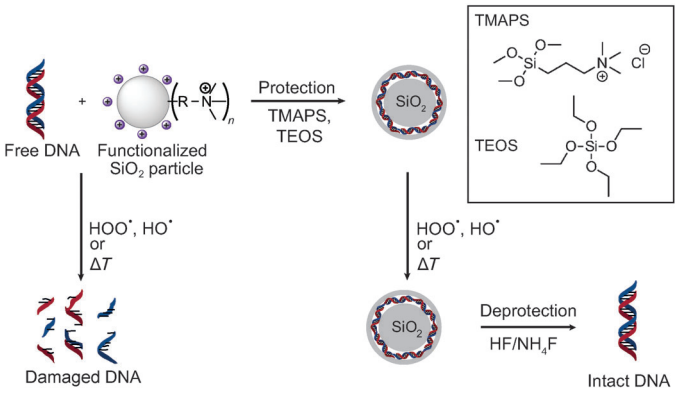
Nucleic acids are the information carriers of all known life forms and can store more information per volume space than magnetic domain or floating gate based technologies (Flash drives $< 1\ TB\,cm^{-3}$; DNA $> 10^8\ TB\,cm^{-3}$). Besides future applications in information storage[1] and DNA-based computing,[2] DNA ismost suited for nanobiotechnology,[3] steganographic messaging,[4] encrypted barcoding, and as an anti-counterfeit tag for consumer goods.[5] However, nucleic acids are sensitive to harsh environmental conditions and elevated temperatures, and biological systems have had to develop elaborate repair mechanisms to maintain information integrity over time (more than 10000 DNA lesions are repaired daily in each human cell).[6] The vulnerability of nucleic acids to hydrolysis (depurination and depyrimidination),[7] oxidation (formation of free radicals mediated by heavy metal ions), and alkylation requires well-controlled DNA storage conditions, ideally dry and at low temperatures.[8] Similarly, ancient DNA (a-DNA) is best recovered from permafrost samples,[9] or in dessicated form from amber[10] and from avian eggshell fossils.[11] Within these fossils a dense diffusion layer (polymerized terpenes or calcium carbonate) separates the desiccated DNA specimen from the environment, water, and reactive oxygen species.

Here we show how the simple encapsulation of DNA in silica particles mimics these fossils and protects DNA from aggressive environmental conditions (Scheme 1). The procedure makes DNA processable at conditions well beyond its biological origin. We demonstrate that silicate and hydrofluoric acid chemistry is compatible with nucleic acid analysis by means of quantitative real-time polymerase chain reaction (qPCR). We also show how silica-protected DNA can be made compatible with injection molding at 200 °C such that polymers and consumer goods can be barcoded.

Silica is well known as a material with high chemical and thermal stability as well as excellent barrier properties and can be synthesized at room temperature by the polycondensation of tetraethoxysilane (TEOS).[12] The incompatibility of TEOS and nucleic acid chemistry (both carrying negative charges under reaction conditions) has been previously solved by the introduction of co-interacting species (positively charged aminosilanes) directing the growth of amorphous silica to the surface of the DNA double helix.[13]



Scheme 1. DNA is protected by immobilization on the surface of cationic charged silica particles onto which a dense silica layer is deposited from TEOS. TMAPS is utilized as co-interacting species to enable the compatibility of the sol–gel processes and DNA. A deprotection procedure based on fluorine chemistry is used to release the encapsulated DNA for subsequent analysis. See Figure S1 for a more detailed description of the experimental procedure.

In an encapsulation approach a standard DNA ladder was first adsorbed to the surface of submicron-sized silica particles ($d = 150$ nm, Figure S2a in the Supporting Information) carrying ammonium surface functionalities. The use of the submicron-sized carriers facilitates the handling of the adsorbed nucleic acids, since separation and washing steps in various liquid media can be accomplished rapidly. In a subsequent step a thin silica layer was grown on the nucleic acid decorated surface utilizing *N*-trimethoxysilylpropyl-*N*,*N*,*N*-trimethylammonium chloride (TMAPS) as co-interacting species and TEOS as silicon source (Figure S1 in the Supporting Information). Although silica surface growth is usually performed under acid or base catalysis, we here chose neutral conditions to prevent the hydrolysis of DNA (Figure S4). Following this relatively slow reaction for four days, we found that a dense silica layer roughly 10 nm thick had grown on the nucleic acids (Figures S2 and S3). Within the particulate structure, it is presumed that each nucleic acid molecule is sandwiched between silica and is suspended by electrostatic interactions with aliphatic ammonium groups (Figure S1).

The encapsulation of DNA in silica has been previously investigated for the formation of complex-shaped nanocomposites;[13,14] however, only if the DNA can be released from the glass spheres unharmed can the stored information be

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utilized for read-out. While silica is unaffected by most chemical reactants at room temperature, it dissolves quickly in hydrofluoric acid (HF) through the formation of hexafluorosilicate ions. Hydrofluoric acid is known as a highly toxic chemical because it can penetrate skin and rapidly damages intracorporal cells and bone.[15] However, aqueous hydrofluoric acid is a relatively weak acid ($pK_a = 3.18$) and it is not expected to damage nucleic acids.[16]

Indeed, it was possible to dissolve the DNA/SiO_2 particles rapidly in buffered oxide etch (HF/NH_4F, a buffered HF solution). Use of low concentrations and minute volumes facilitated safe working routines. Following this procedure the presence of the DNA could still be proven by gel electrophoresis (Figure 1, lane 2); no quantitative differences such as

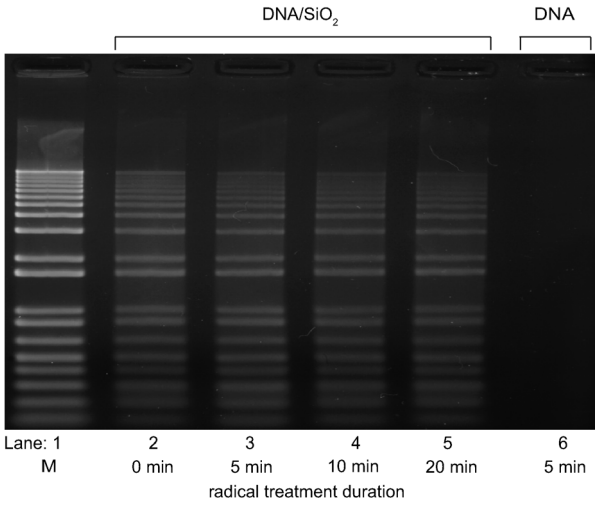

Figure 1. SiO_2-protected DNA ladder survived treatment with copper-generated radicals for up to 20 min (lanes 2–5). For comparison, nonprotected DNA treated under the same conditions was completely destroyed (lane 6). M: DNA ladder plus (Invitrogen).

strand breaks were observed (cf. unprocessed DNA ladder; Figure 1, lane 1). In order to test the completeness of the coating and its protective properties, the particles were treated with highly aggressive heavy metal and hydrogen peroxide containing solutions which induce the formation of reactive oxygen species (ROS).[17] Under the conditions applied (230 μM $CuCl_2$, 6.6 mM H_2O_2, 1.3 mM ascorbic acid) unprotected nucleic acids were destroyed nearly instantly (see gel electrophoresis; Figure 1, lane 6) indicating the aggressiveness of the method. The DNA encapsulated in the particles, however, was hardly affected by the treatment, even after 20 min (Figure 1, lanes 3–5).

However, the mere presence of sharp bands on the electrophoresis gel does not prove the amplificability of the DNA. For this purpose a known DNA amplicon (113 bp, see the Supporting Information for sequence) was encapsulated into the particles by the procedure described above. Following the simulation of various environmental conditions and upon dissolution of the particles in HF/NH_4F, the induced damage of the DNA could be monitored by qPCR analysis (Figure 2; see the Supporting information for details on the full experimental procedure).

For a direct comparison, solutions with similar DNA concentrations were prepared from the unprotected DNA amplicon and the SiO_2-protected DNA amplicon (same μg DNA mL^{-1}). Firstly these solutions were stressed with radical oxygen species as described above. Similar to the experiments on the DNA ladder, the free amplicon was nearly fully degraded by the radical oxygen species after a few minutes whereas most of the SiO_2-protected amplicon withstood the treatment (Figure 2 a). Therefore, even the very thin silica shell (ca. 10 nm thick; Figure S2 in the Supporting Information) prevented the diffusion of radical oxygen species to the encapsulated DNA. Based on the data point at 0 min the capacity of the particles can be calculated as

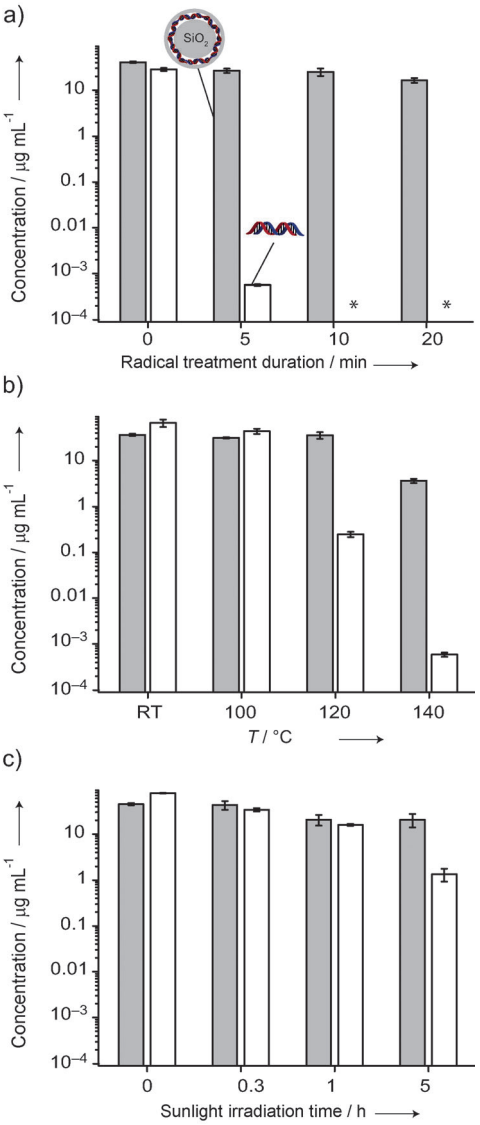

Figure 2. qPCR analysis of DNA/SiO_2 stability compared to that of unprotected DNA. Dispersions of particles (17.5 mg particles mL^{-1}) and free DNA amplicon (40 μg mL^{-1}) were treated under various conditions. Gray bars represent protected DNA, white bars free DNA; star (*) indicates data below the detection limit ($< 10^{-6}$ μg mL^{-1}). a) Treatment with copper-generated radicals for durations of up to 20 min; b) heat stability at temperatures between 100 °C and 140 °C for 15 min (pressurized); c) stability following simulated sunlight irradiation at 1000 W m^{-2} for up to 5 h.

(2.5 ± 0.5) μg DNA per mg of particles. This is equivalent to approximately 75 dsDNA amplicon molecules per individual particle. In our procedure every DNA molecule is protected by a dense diffusion layer, similar to the encapsulation of DNA in fossils or spores.[18] Since the most prominent ROS ($OH^{\bullet}$ and $OOH^{\bullet}$) are very small molecules with high diffusion constants, it appears that the silica shell acts as a hermetic diffusion barrier and protects equally well against larger chemical reactants and enzymatic DNA digestion by nucleases.

Experiments on heat stability gave similar results (Figure 2b): Less than 2% of the DNA in DNA/SiO_2 particles was degraded by wet heat treatment (120°C) for 15 min, conditions at which 99.5% of the free DNA was destroyed. At higher temperatures the degradation of nonprotected DNA is significantly accelerated[19] and assisted by singlet oxygen species.[20] A further decay-promoting factor is UV irradiation. Whereas nonprotected DNA was degraded under sunlight irradiation (Figure 2c) after five hours, the decomposition is slower for DNA encapsulated in silica. This effect is somewhat unexpected, as amorphous silica is known for its good UV transparency (>60% transparent at 170 nm) but may be explained in terms of the light scattering of submicron-sized particles at low wavelengths as described by the Mie theory.[21] Since the particles are in the size range of the wavelength of UV light, light cannot pass through the particle and is mostly scattered from the surface of the individual particles, protecting the DNA encapsulated therein. Further stability against UV irradiation may be gained by incorporation of the particles into an UV-absorbing matrix as described below.

Now having a storage format for DNA, which can withstand environmental conditions and elevated temperatures, we envisaged the barcoding of products. The nearly infinite variability of possible codes, the possibility of encryption, the low-cost analysis, and the ultralow detection limit (qPCR) make DNA an ideal coding system for the tagging of products. DNA barcoding of goods of biological origin (e.g. foodstuffs, cotton, animals) has been utilized for many years for the investigation of market substitution, fraud, and counterfeit.[22] In this context, chromosomal or mitochondrial DNA can be utilized as the target and the information integrity over time within the original cells is a minor issue. Translation of this concept to nonbiological systems has been attempted but currently suffers from the instability of free nucleic acids under environmental conditions[23] and the elevated temperatures required during material processing. In contrast to previously developed systems for DNA protection, including mesoporous silica,[24] DNA complexes with polycations[25] and layered double hydroxides,[26] which offer stability against enzymatic digestion, the nonporous particles presented here also protect DNA against ROS and heat-induced degeneration. This makes these particles most suited as marker particles to barcode consumer goods and products with artificial DNA codes. Good heat stability is of particular importance if DNA is to be applied as a barcoding system for plastics, where traditional processing by injection molding requires elevated temperatures. In an attempt to utilize the particles developed here for the barcoding of polymers, particles containing a known amplicon (pBluescript II KS, MCS, 238 bp) mixed within nonamplifying DNA (DNA ladder) were incorporated into commercial polysulfone (Figure S6 in the Supporting Information) and polyvinylchloride (PVC) blends (Figure 3). In order to investigate the required amounts, various loadings from $\delta = 0.1$ ppm to 0.2 wt% were tested ($= 100\ \mu g\,kg^{-1}$ to $2\ g\,kg^{-1}$; see Table S1 in the Supporting Information). Whereas the highest loadings were chosen to show that the particles do not influence the optical properties of the polymers, lower concentrations were utilized to depict the limitations of the method. The two polymers were chosen as representative examples for high-value and high-volume thermoplasts, respectively. Polysulfone-DNA/SiO_2 composites were formed by solvent casting and processed to thin films. Even at higher loadings (0.2 wt%) the optical properties of the films were not affected and transparency could be maintained (Figure S5). Following solvent dissolution of the polysulfone films, recovery of the particles by centrifugation, and subsequent dissolution of the particles in HF/NH_4F (Figure S7), the identity of the polymers could be proven by qPCR analysis of the amplicons (Figure 3 and Figure S6).

To test the heat stability of the encapsulated DNA/SiO_2 particles within the polymers (dry heat), polysulfone and PVC samples were treated for 15 min at temperatures up to 200°C (Figure 3a, Figure S6). Although these temperatures resulted

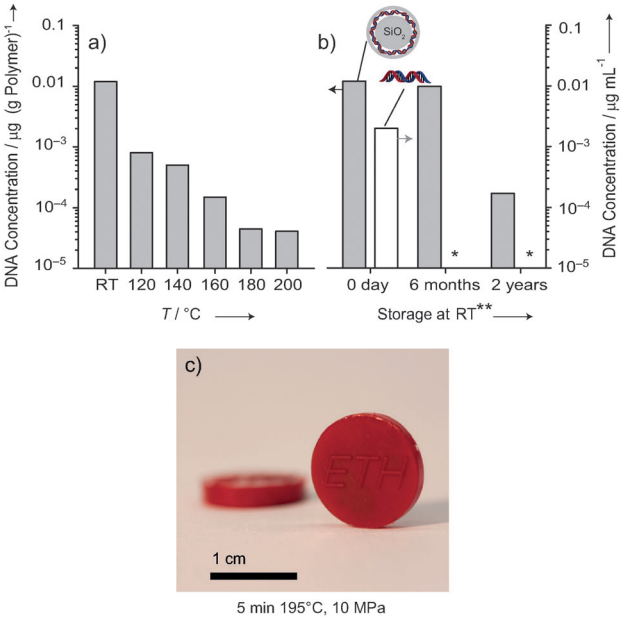

Figure 3. Stability of DNA/SiO_2 in PVC ($\delta = 350$ ppm DNA/SiO_2). a) qPCR-quantifiable concentration of DNA amplicon in the polymer as a function of temperature (15 min); b) simulated long-term stability of DNA/SiO_2 in PVC (gray bars, left axis) and unprotected DNA (white bars, right axis) in water at RT following ASTM F1980 (**Accelerated Aging of Sterile Medical Device Packages, Q10 = 2); star (*) indicates data below the detection limit ($< 10^{-6}\ \mu g\,mL^{-1}$); c) hot-press-processed PVC-DNA/SiO_2 composite ($\delta = 350$ ppm particles) processed at 195°C and 10 MPa for 5 min with a quantifiable concentration of 10^{-3} μg DNA g^{-1} polymer following the treatment (detection limit is 10^{-6} μg DNA g^{-1} polymer). Particles contain both pBluescript amplicon and DNA ladder (1:30, see Section 3.3 in the Supporting Information). During quantification with qPCR only the concentration of the amplicon is measured and reported here.

in a considerable loss of quantifiable DNA, the DNA packed within the particles could still be amplified by qPCR and used to unequivocally identify the polymer. The processing of DNA at these unusually high temperatures should make it possible to apply nucleic acid barcoding with polymer injection molding. In order to test this, PVC containing DNA/SiO_2 particles ($\delta = 350$ ppm) was processed by a hot-press ($T = 195\,^{\circ}C$ for 5 min) to give a three-dimensional pill (Figure 3c). Following a two-step extraction protocol (Figure S7 in the Supporting Information), the nucleic acids could be freed from the polymer, and the polymer could still be unequivocally identified utilizing qPCR. Similar results were obtained by simulating long-term aging of protected DNA in PVC following the ASTM F1980 norm. Successful amplification of DNA was achieved after storage at 60 °C for 2 months (equivalent to room-temperature storage for 2 years, see Figure 3b).

As can be seen from the above examples, the protection of DNA by encapsulation and the subsequent deprotection by treatment with HF solutions is chemically orthogonal to the biochemical processing and analysis of nucleic acids, as well as to the processing of ubiquitous polymer-based consumer goods. The protection step affords DNA stability beyond that of its most familiar applications in biological systems and extends DNA barcoding to materials of nonbiological origin. The combination of protected nucleic acids and ultrasensitive biochemical analysis by qPCR makes it possible to prepare chemically stable tracer particles, carrying unique codes with very low detection limits. Our work may lead to new materials for anti-counterfeiting and identifying labeled objects, product streams, and supply chains.

Experimental Section

General synthesis of protected DNA: Ammonium-functionalized silica particles (35 μL, 50 mg mL^{-1}) were mixed with 10 μL of double-stranded DNA (1 mg mL^{-1}, various DNA amplicons as well as DNA ladder were used, see the Supporting Information) and 1 mL of water. After intense mixing and centrifugation of the particles for 2 min at 21500 g, the supernatant was discarded and the particles were resuspended in 1 mL of H_2O. Following two additional washing cycles, the centrifuged particles were dispersed in 0.5 mL of water, after which the co-interacting agent (TMAPS, 50% in MeOH, 0.6 μL) was added while the suspension was stirred at room temperature. Tetraethoxysilane (TEOS, ≥99.0%, Aldrich, 0.6 μL) was added to the vigorously stirred suspension and the mixture was allowed to react at room temperature for 4 h, after which additional 4 μL of TEOS were added. The mixture was stirred (900 rpm) for 4 days.

DNA release: For the extraction of the encapsulated DNA, 10 μL of the particle suspension was added to 40 μL of a buffered oxide etch solution (HF/NH_4F, 0.34 g NH_4F + 10 g HF (1% in water)).

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