A New Marking System for Leather Based on Encapsulated DNA

by

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Abstract

The use of synthetic DNA as a marking system is a new traceability concept in the leather industry, especially for supplier and batch tracing. DNA is outstandingly suited for the usage as a marking system because of its code diversity, invisibility and doubtlessness. However, DNA labeling is a great challenge for products exposed to DNA damaging influences during their production, such as acidic pH, elevated temperatures in combination with high humidity or sunlight radiation. Leather is such a product. We attached single-strand DNA (ssDNA) to hydroxyapatite and enhanced the stability of these DNA particles by encapsulation in polystyrene-co-divinylbenzene (PS-DVB) microcapsules. Furthermore, the ssDNA containing microcapsules were improved with functional groups on the surface of the capsule to irreversibly attach them to the collagen matrix of leather by chrome tanning. Laboratory scale tests using acidic conditions as well as elevated temperatures in the presence of high humidity showed that the stability of the leather marking system was enhanced. Marking trials were conducted in crust leathers, and the light fastness of these labeled crusts were tested. The results indicate that encapsulated DNA-hydroxyapatite-particles are more stable at sunlight radiation than non-encapsulated DNA. These marking trials showed that the system could be a suitable leather marking system in the leather industry to establish a powerful supplier and batch tracing.

Introduction

Current marking systems for leather hides are stamps or embossed numbers. Both are based on punched holes. Leather cuttings produced during leather processing are marked by bar code stickers on their back side. The labeling with bar codes as well as stamps show some major draw backs because these labels often get lost during subsequent production steps.

During leather making, a marking system is exposed to extreme and contrary environmental conditions. During tanning, pH-thresholds up to pH 2.0 are reached. Upon drying, the semi-finished leather is exposed to temperatures up to 80°C. In finished leather, a leather marking system can also be exposed to environmental influences, such as increased humidity, sunlight radiation, or slightly acidic pH values caused by the leather itself. As a biopolymer, DNA is outstandingly suited for supplier and batch tracing in leather industry. The base sequence of a DNA molecule enables a code diversity, which is hardly achievable by other techniques. Gavent and co-workers adopted this idea for a new leather marking system by integration of synthetic DNA during the leather finish.[1] However, the authors did not measure a long-term stability of the DNA.

The long-term DNA stability is of great importance, because native DNA is damaged under the conditions mentioned above[2-5] (for review see [6]). These extreme impacts lead to DNA fragmentation and damage the base sequence; hereby causing a loss of stored information in a DNA based marking system. If DNA is proposed as an invisible barcode for leather protection, it has to be protected. Based on an idea of Bohrisch et al.[7] we embedded ssDNA into a hydrogel sphere and coated it with a shell of cross linked polystyrene in a previous work.[8] In this work, ssDNA was embedded in a polyacrylamide gel core which was then coated by PS-DVB microcapsules. The encapsulation led to protection of the ssDNA inside of the microcapsules against an acidic pH of 2.0. A schematic overview of the basic structure of the produced leather marking system is shown in Figure 1.

It was noticed by Lindhal[6] and also demonstrated by our laboratory[9] that DNA can be stabilized against higher temperatures by its attachment to hydroxylapatite. In the present work, we combined these two approaches of DNA protection, first against acidic pH by encapsulation and second against elevated temperatures by attaching DNA to a

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stabilizing mineral phase, and developed a new optimized marking system for leather products. The developed marking system consists of PS-DVB microcapsules displaying a ratio of styrene and divinylbenzene of 1:1 (50%) and containing ssDNA attached to hydroxyapatite.

In addition, we synthesized PS-DVB microcapsules with functional groups on the surface of the capsule to (a) detect them microscopically by fluorescence (Rhodamine B) and (b) ligate them to the collagen matrix (carboxyl groups). We choose carboxyl groups, because chromium forms stable complexes with carboxyl side groups of the leather's collagen matrix during chrome tanning.

The stability of our new leather marking system was tested by standard testing procedures for leather, e.g. alternating climate test and test for light fastness. The changed stability of encapsulated ssDNA in contrast to non-encapsulated ssDNA was detected by quantitative real-time polymerase-chain-reaction (qPCR).[9-10]

EXPERIMENTAL

Materials

All solvents except Isopar M (Exxon Mobil, Machelen, Belgium) were supplied by Sigma-Aldrich and dried by standard procedures. Span 80, Tween 85, ethylenediamin tetraacetic acid (EDTA), styrene, divinylbenzene, 4-vinylbenzyl chloride, acrylamide, rhodamin B base, piperazine, trimethylaluminium, dimethylmalonate and N,N'-methylenebisacrylamide were provided by Sigma-Aldrich and used as received. Hypermer 2296 (Croda, Goole, UK), 2, 2'-azobis-(2,4-dimethylvaleronitrile) (V-65) and 2,2'-azobis-(N,N'-dimethyleneisobutyramidine) dihydrochloride (VA-044) (both Wako Chemicals, Neuss, Germany) were commercial grade and used as received.

Synthetic single-strand DNA (ssDNA; 50 base pairs long, named 1/50) and the associated primers for PCR analysis were designed manually and were obtained from Life Technologies GmbH (Darmstadt, Germany). For sequence information see section "supplementary materials" or Stenzel and Meyer.[9]

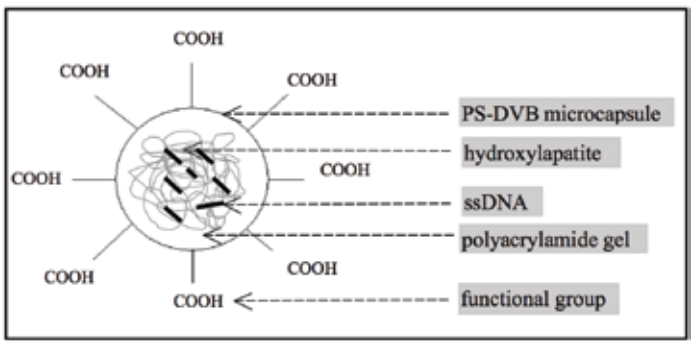


Figure 1. Overview of the basic structure of the leather marking system.

Synthesis of Functional Monomers

Rhodamine B monomer. Synthesis was performed based on rhodamine B base which was transformed to corresponding piperazine amide as published elsewere.[11] In summary, 1.5 g (2.85 mmol) of rhodamine B base were dissolved under argon in 15 mL dry CH_2Cl_2 in a 50 ml 2-necked flask. At 0°C (ice cooling), 0.54 g (6.84 mmol) of dry pyridine and 0.6 g (5.7 mmol) of freshly distilled methacryloyl chloride were added consecutively. Afterwards, this mixture was stirred overnight at room temperature (RT), diluted with 200 mL of CH_2Cl_2 and extracted with 200 mL aqueous saturated $NaHCO_3$-solution. The aqueous phase was extracted four times with 100 mL CH_2Cl_2 and then saturated with NaCl and extracted again with 100 mL isopropanol/CH_2Cl_2 (1:4). The original organic phase and the organic extracts were unified and dried with Na_2SO_4, filtered and freed from residual organic solvents at 10^{-3} mbar for 3 days. NMR data (in ppm, $CDCl_3$): (6.8-7.8 (aryl, 10H), 5.2 (vinyl, 1H), 5.0 (vinyl, 1H), 3.6 (8H, N-CH_2), 3.4 (8H, N-$CH_{2,Piperazin}$), 1.9 (3H, $CH_{3,Methacr}$), 1.4 (12H, CH_3). The overall yield was 20%. The rhodamine B monomer (content ~ 90% according to ^{1}H-nmr) was used without further purification.

"Carboxyl monomer" (1): 4-Vinylbenzylmalonic acid. The preparation was carried out according to a procedure described elsewhere.[12] Shortly, Sodium (2.51 g, 109 mmol) was dissolved in absolute ethanol (100 mL). When the evolution of hydrogen gas ceased, dimethyl malonate (43.3 g, 328 mmol) was added quickly, followed by a dropwise addition of 4-vinylbenzylchloride (16.6 g, 109 mmol; containing BHT as an inhibitor). After the addition was complete, the reaction mixture was stirred for 3 h at 50°C and then poured into water. The mixture was extracted three times with dichloromethane and the collected extracts were dried over magnesium sulfate. Dichloromethane was removed using a rotovap and the residue was distilled in vacuumed to obtain dimethyl (4-vinylbenzyl) malonate (18.4 g, yield 68%). The saponification was carried out in refluxing aqueous ethanol (20 mL water, 40 mL ethanol) under N_2 with potassium hydroxide (14.8g). The reaction was followed by thin layer chromatography (silica; hexane/ethyl acetate/acetic acid 4/12/1) and found complete within 2.5 h. All volatiles were removed using a rotovap. The obtained residue was treated with dilute hydrochloric acid. The product was extracted with five portions of diethylether, which in turn was washed with brine and dried with sodium sulfate. Filtration and removal of ether left a product sufficiently pure for the polymerizations described below. NMR data (in ppm, acetone-d_6): 7.38 (aryl, 2H), 7.27 (aryl, 2H), 6.72 (vinyl, 1H), 5.76 (vinyl, 1H), 5.19 (vinyl, 1H), 3.71 (CH, 1H), 3.17 (CH_2, 2H). The overall yield was 62%.

"Carboxyl monomer" (2): 4-Vinylphenylacetic acid. Synthesis was carried out according to a patent application.[13] In detail: Magnesium turnings (2.63 g, 0.108 mol) were placed in a 250 mL three-neck round bottom flask fitted with a nitrogen inlet, an addition funnel and a reflux condenser with a bubbler. The

complete apparatus was dried in a flow of nitrogen using a heat gun. When cold, ethyl ether (30 mL, freshly dried over benzophenone/Na/K) was transferred to the flask. The addition funnel was charged with vinylbenzylchloride (15.26 g, 0.1 mol) and 50 mL of ethyl ether. Using a syringe, about 0.5 mL dibromoethane were added directly to the magnesium turnings without agitation. When the formation of ethylene ceased, the content of the dropping funnel was slowly added to the flask keeping the mixture at a gentle boil. During the reaction, the solution turned yellow-greenish. After the addition was finished, the flask was placed in a 40°C water bath for two hours to complete the reaction. In the meantime, coarse pieces of dry ice (~150 g) were crushed in a beaker under nitrogen to prevent the condensation of moisture and the Grignard solution was poured swiftly with agitation onto it. About 100 mL of diethylether were added to the mixture, followed by hydrochloric acid to liberate the vinylphenylacetic acid. The ether phase was transferred to a separation funnel and reacted with aqueous potassium carbonate until basic. The remaining ether phase containing the side product (1,2-di (4-vinyl phenyl) ethane) was discarded, the aqueous phase was acidified and extracted with ether. The purity of the reaction product was tested at this stage by thin layer chromatography (hexane/ethyl acetate 20/1, R_f product: ~0.08, R_f side product: 0.82) and found satisfactory. Ether was removed from the product by rotary evaporation after adding a pinch of BHT as inhibitor. NMR data (in ppm, $CDCl_3$): 7.38 (aryl, 2H), 7.24 (aryl, 2H), 6.70 (vinyl, 1H), 5.74 (vinyl, 1H), 5.25 (vinyl, 1H), 3.64 (CH_2, 1H). The overall yield was 49%.

Synthesis of PS-DVB Microcapsules

Different variants of capsules named **C1 – C6** (Table I) were synthesized in two steps.

Step 1 (Inverse emulsion polymerization of the acrylamide core): An aqueous phase containing acrylamide (1.03 g, 14.5 mmol), N,N-methylenebisacrylamide (11.2 mg, 1.7 mmol), EDTA aq. 1% (1.67 g), (hydroxyapatite/ssDNA 1/50 (20µg)) and water (19.34 g) was mixed with an organic phase containing Isopar M (6.51 g), Span 80 (0.55 g), Tween 85 (1.10 g), Hypermer 2296 (0.07 g) for 1 min at 3000 rpm. Transfer of the reaction mixture into a nitrogen flushed lab reactor at 45°C was followed by addition of VA-044 (11.5 mg) dissolved in 1 mL of water. After 4 h, a second portion of VA-044 (11.5 mg) in water was added. The polymerization was stopped after 24 h.

Step 2 (Microencapsulation): The procedure for encapsulation was as follows: A solution of Isopar M (37.19 g), Span 85 (2.97 g), Tween 61 (1.61 g), styrene, divinylbenzene, rhodamin B methacrylate, 4-vinylphenylacetic acid and 4-vinylbenzylmalonic acid (for proportions of all monomers see Table 1) was mixed for 1 min at 3000 rpm. Then, the gel particles of step 1 (32 g) were added and the mixture was stirred again for 1 min at 3000 rpm. The reaction mixture was filled into a stirred (200 rpm) lab reactor, purged with nitrogen and heated to 45°C. Initiator V-65 (2.25 g; 16% in toluene) was quickly added. In the same way, further portions of V-65 were added after 4 and 8 h. After 24 h, the polymerization was stopped. In order to remove impurities and most of the surfactants, the coated particles were allowed to settle and washed 3 times with hexane.

Synthesis of PS-DVB Microcapsules Containing Functional Groups

The aim of this part was to obtain capsule monomer additives, which are easily accessible from inexpensive precursors. Rhodamine B methacrylate was chosen as a polymerizable

TABLE I
Composition of capsule monomers.

Capsule	ssDNA/ hydroxyapatite	Oil phase composition (g)			
		Styrene	Divinyl-benzene	Rhodamin B monomer	Carboxylic acid monomer
C1	-	4.83	4.83	0.025	0
C2	-	4.59	4.59	0.025	0.48 (1)
C3	-	4.59	4.59	0.025	0.48 (2)
C4	+	4.83	4.83	0	0
C5	+	4.83	4.83	0.025	0
C6	+	4.59	4.59	0	0.48 (1)

(1)… Carboxyl monomer: 4-Vinylbenzylmalonic acid (5% COOH-monomer)
(2)… Carboxyl monomer: 4-Vinylphenylacetic acid (10% COOH-monomer)

fluorophore due to a convenient synthesis route without the use of chromatography (Scheme 1).

Two different routes were followed to obtain carboxyl groups containing additives. Commercial chloromethylstyrene was the starting monomer. Thereon, acid groups are either obtained from the alkylation of the CH acidic malonic ester and subsequent hydrolysis (1) or the Grignard synthesis with CO_2 as the nucleophile (2) (Scheme 2).

The inverse emulsion polymerization was performed using acrylamide, crosslinking agent methylenebisacrylamide and water-soluble initiator VA044. In this way, ssDNA partly attached tohydroxyapatite was embedded in a polyacrylamide gel. For the stabilization of the gel particles in the solvent, Isopar M (high boiling mixture of iso paraffins) and a mixture of surfactants of the sorbitan type (hydrophilic-lipophilic balance, HLB = 8.6) were used. The hydrogel particles were used without further purification. After that, ssDNA-containing gel particles were covered with a cross linked polystyrene shell by a precipitation polymerization of styrene and divinylbenzene and a functional compound such as polymerizable fluorophore rhodamine B and/or carboxyl group containing monomer. Rhodamine B acts as a sensitive marker to trace capsules e.g. in washing procedures. On one hand, the capsules protect their insides against mechanic attack and partly avoid diffusion exchange of solvents. On the other hand, incorporated carboxyl groups promote a fixation of the capsules to collagen or leather. A simple procedure to prepare hydrophilic core/hydrophobic shell particles via inverted emulsions was first proposed by Ruckenstein and co-workers.[14] Following that, the resulting inverse emulsion of step 1 is mixed with an Isopar phase containing the monomers, a mixture of stabilizers (HLB ~ 4.5) and an oil soluble initiator. Due to the insolubility of cross linked polystyrene in Isopar the polymeric material precipitates on the surface of the hydrogel. The particles are easy to purify by settling and decanting several times.

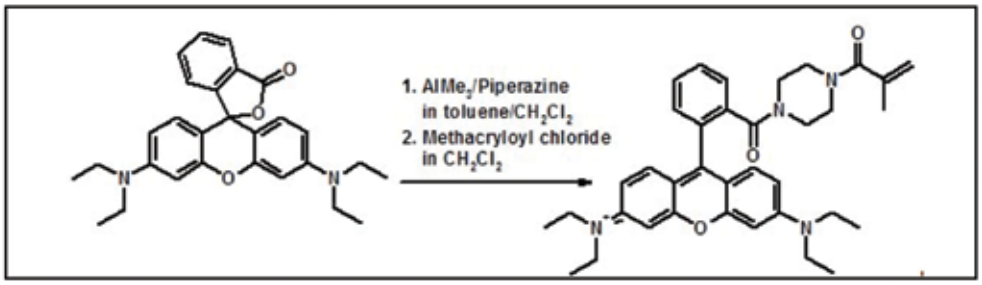
Marking Trials with Non-encapsulated DNA as Part of the Base Coat

Non-encapsulated synthetic ssDNA 1/50 (for sequence information see Stenzel and Meyer[9]) was mixed with the base coat of a finish recipe. The tagged base coat was used to treat three crust leathers of the same origin. The trials were arranged at the Hewa Leder Company (Freiberg, Germany). Every coated square meter was marked with 212 µg ssDNA. Afterwards, the marked leathers were covered with two further standard finish layers.

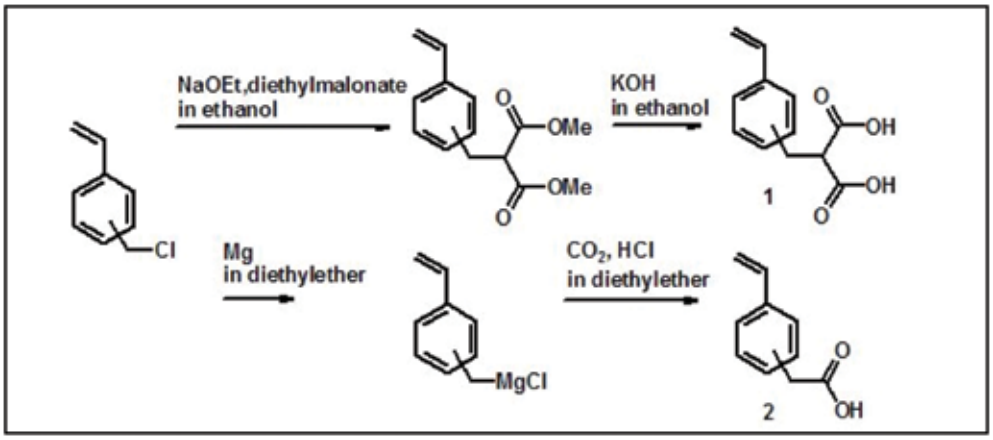
A sample was kept for both the alternating climate test and the test for light fastness. As a negative control, one crust was finished without the addition of ssDNA (Figure 2).

Alternating Climate Test

The alternating climate test used in this study is established in the automotive industry and consists of the following cycles (20 repeats): 4 h storage at 40°C/ 90% air humidity, 2 h heating to



Scheme 1: Synthesis of polymerizable fluorophore.



Scheme 2: Synthesis of carboxyl groups containing monomers.

120°C (reduction of air humidity in 80 min to 10%), 4 h storage at 120°C, 2 h cooling to 40°C and increase of air humidity to 90%. For the test, a WK 340 / 40 (Weiß Umwelttechnik, Reiskirchen-Lindenstruth, Germany) was used.

Test of Light Fastness

The test of light fastness was conducted according to DIN EN ISO 105-B02. This test simulates sunlight radiation with 360 000 kJ/m^2 in 200 h. The used equipment was a Suntest CPS+ (ATLAS, Linsengericht-Altenhaßlau, Germany).

DNA Isolation from Base-coat-marked Leathers Containing ssDNA

The ssDNA was isolated from the base coat with the MasterPure™ Complete DNA and RNA Purification Kit (epicenter) according to the manufacturer's instructions, except that the digestion step with proteinase K was extended to 2h.

Laboratory Scale Test: pH 2

Sample **C4** was aliquoted (25 mg each) and was incubated in 200 µl Theorell-Stenhagen-Buffer (TSB, pH 2.0; 33 mM citric

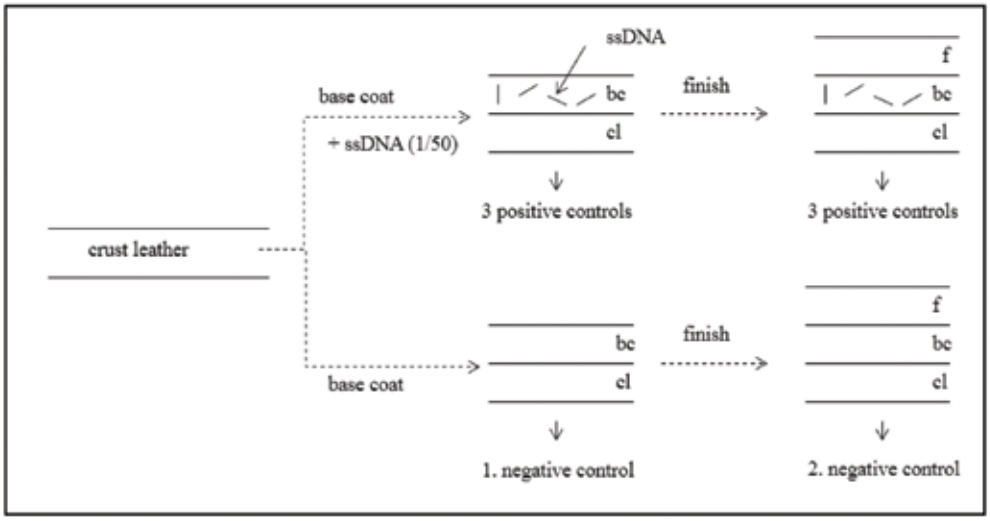


Figure 2. Overview of the reference leather production with non-encapsulated ssDNA (bc- basecoat, cl- crust leather, f- finish.)

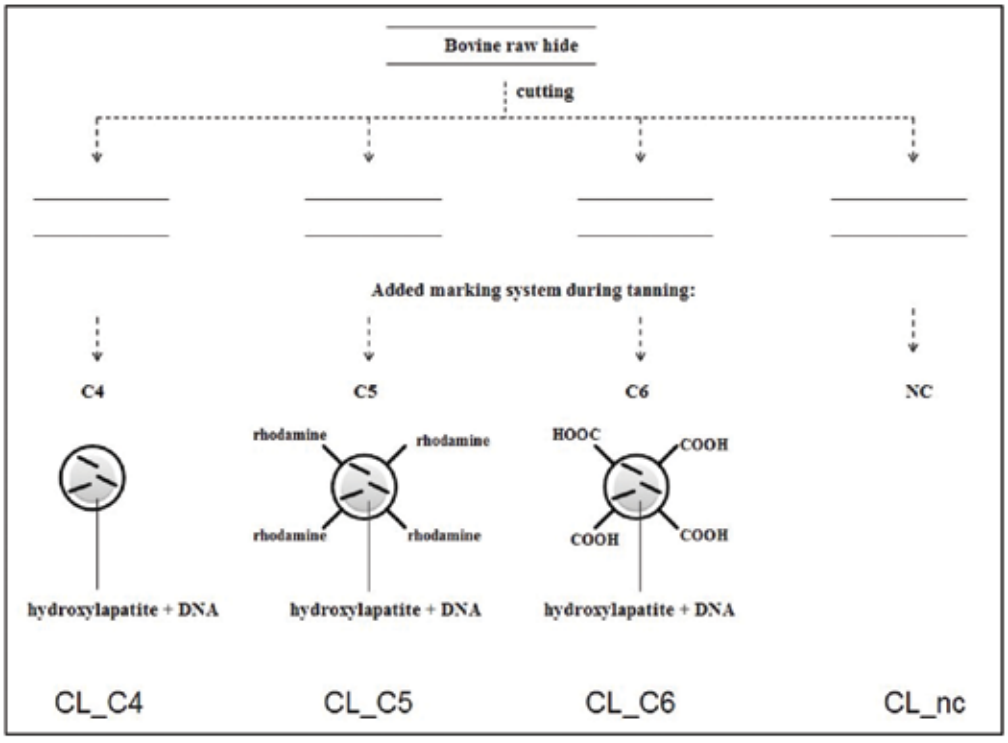


Figure 3. Overview of the used labeled crust leathers and the non-labeled crust leather. CL_C4, CL_C5 and CL_C6 are the labeled crust leathers; CL_nc is the non-labeled crust leather. (nc- negative control.)

acid, 33 mM phosphoric acid, 57 mM boric acid, 34,3 mM sodium hydroxide; pH was adjusted with 0,1 M HCl). For each time period (0, 4, 7, 11, 14, 21, 25 days), three aliquots of **C4** were incubated in the dark at room temperature. After sample treatment, the acidic samples were neutralized with TSB (without HCl).

Laboratory Scale Test: 80°C

Three aliquoted samples of **C4** (25 mg each) were incubated in 400 µl bidest water at 80°C for two time periods each. The first time period was 0, 1, 2, 3, 4, 7 days, the second time period was 0, 1, 2, 3 days. Afterwards, the loss of water was analyzed by weighing and all samples were filled up with bidest. water to 400 µl.

Marking Trials with Encapsulated ssDNA

Samples **C4**, **C5** and **C6** were implemented as markers during the manufacturing of a wet blue. Salted bovine rawhide was unhaired and cut into 4 equal A4 pieces. The pieces were transferred into wet blues using a standard technology. 10 g of each sample were added to one of the four skin pieces during tanning. The fourth wet blue served as negative control without marking (Figure 3). Subsequently, the wet blues were finished to produce crust leather and dried. Three 5 cm x 5 cm pieces were cut from each crust leather and used for the alternating climate test and the test of light fastness.

Isolation of PS-DVB Microcapsules from Leather

About 50 mg of leather were incubated for 2 h at 56°C in 1 M NaOH. Afterwards the NaOH was neutralized with HCl.

DNA Isolation from PS-DVB Microcapsules

The dispersed microcapsules were flash frozen for 10 sec in liquid nitrogen. Afterwards, phosphate buffer was added to reach an end concentration of 300 mM. Glass beads (Ø 0.1-0.25 mm) were added, the sample was vortexed for 15 min and afterwards centrifuged for 2 min/ 4000 rpm. The ssDNA was isolated from the obtained solution using either the peqGOLD MicroSpin Cycle-Pure Kit (peQlab, Erlangen) (laboratory scale test) or the MasterPure™ Complete DNA and RNA Purification Kit (epicenter, Wisconsin) (marking trials with encapsulated DNA) according to the manufacturer's instructions.

Neutralized samples were digested and the released ssDNA was analyzed by qPCR for total ssDNA damage.

Quantitative Real-time PCR (qPCR) and Analysis of qPCR-data/statistics

The qPCR assay, the analysis of the qPCR-data and the statistics were adopted from a previous study.[9] In short: Total DNA damage was detected by amplifying the treated and non-treated DNA with qPCR based on SYBR Green I dye detection. qPCR conditions are described in detail in the section "supplementary material". Checked data were used for relative quantification based on a standard curve method in separate tubes (instructions from user bulletin no. 2, PE Applied Biosystems) in duplicates. The relative amplification ratio R_A[10] is described by the ratio of the measurable starting quantity of treated DNA (A_D) to the measurable starting quantity of untreated DNA (A_0): $R_A = A_D/A_0$. For identification of outliers, statistical analysis was carried out with Dixon's Q-test (n=3; α= 0.05). The amplification ratio behavior of a damaged template matched a natural decay over the time corresponding to a first order reaction rate $R_A = R_0 \exp(-kt)$, where R_0 is the amplification ratio of non-damaged template and k is the apparent rate constant. The rate constant k was calculated by linear regression using SigmaPlot 11.0. The half-life $t_{1/2}$ was determined with $k = \ln 2/t_{1/2}$. The calculated rate constant and the half-life for ssDNA are only apparent constants, because they represent the accumulation velocity of DNA damage, which is not detectable with qPCR. The DNA damage, which is amplifiable, has no influence in this method.[9,10]

Hide Powder Tanning

0.1 g of sample **C1** and sample **C2** were added to 0.5 g wet Freiberger hide powder, respectively. After 20 min of shaking, a surfactant (commercially available dish liquid) was added and the solution was shaken again for 20 min. Afterwards, the pH was adjusted to 3.0 with 8% NaCl and 0,1% formic acid. The hide powder was tanned for 3 h with 10% Basochrom 50 (Völpker Montanwachs GmbH, Völpke). Afterwards, the pH was adjusted to 4.0 – 4.2 with $NaHCO_3$. The liquid was separated by vacuum filtration. The produced hide powder pellets were washed four times with water to remove unbound sample. One wash step consisted of 5 min shaking in water and subsequent centrifugation. Afterwards, the pellets were dried and analyzed by fluorescence microscopy.

Fluorescence Microscopy and Scanning Electron Microscopy (SEM)

Hide powder pellets as well as the water obtained from the washing step after tanning were analyzed with an Axio Scope A1 (Zeiss, Jena, Germany). PS-DVB microcapsules were imaged with SEM on a Philips XL30 (ESEM).

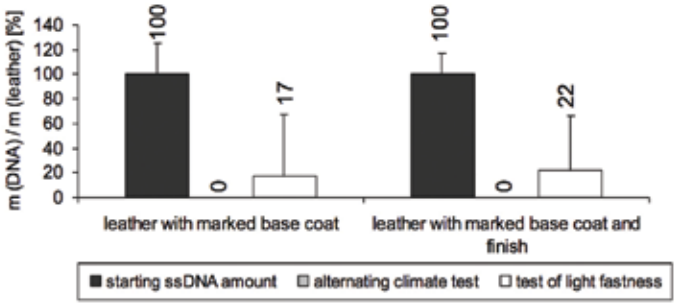


Figure 4. Measurable DNA content in the base coat before and after the alternate climate test and the test of light fastness. Every bar comprises six independent values generated from three leathers.

RESULTS AND DISCUSSION

In previous investigations, it was shown that a PS-DVB microcapsule with a ratio of styrene and DVB of 50% shows the best enhancement of ssDNA stability at pH 2.0.[8] In other studies, the stability of ssDNA at high temperatures was enhanced by the attachment to hydroxyapatite.[6,9] As a consequence, we synthesized 50% PS-DVB microcapsules containing ssDNA attached to hydroxyapatite to use them as a marking system for leather. Furthermore, the surface of the microcapsules was functionalized with carboxyl groups to attach the marking system to the collagen matrix of leather during tanning.

Marking Trials with Non-encapsulated DNA (reference)

To generate reference values, three leathers were marked with non-encapsulated synthetic ssDNA by mixing it into the base coat. Two of these leathers were subsequently finalized with intermediate and topcoat (Figure 2). All three leathers were

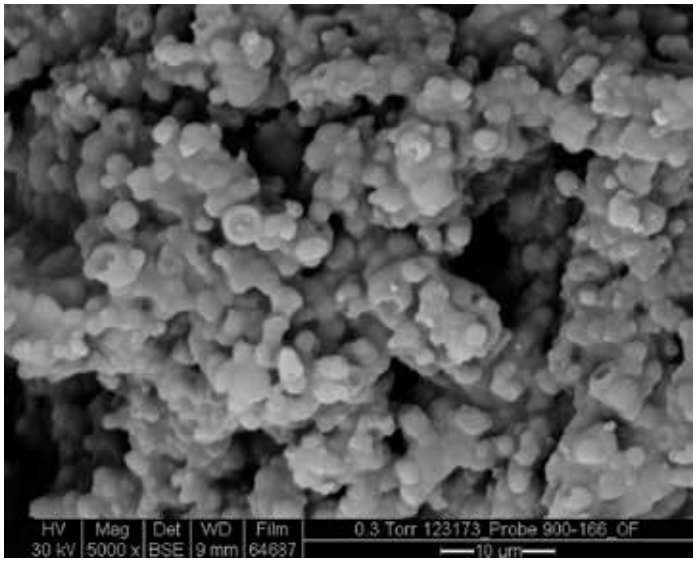


Figure 5. SEM-picture of sample C4.

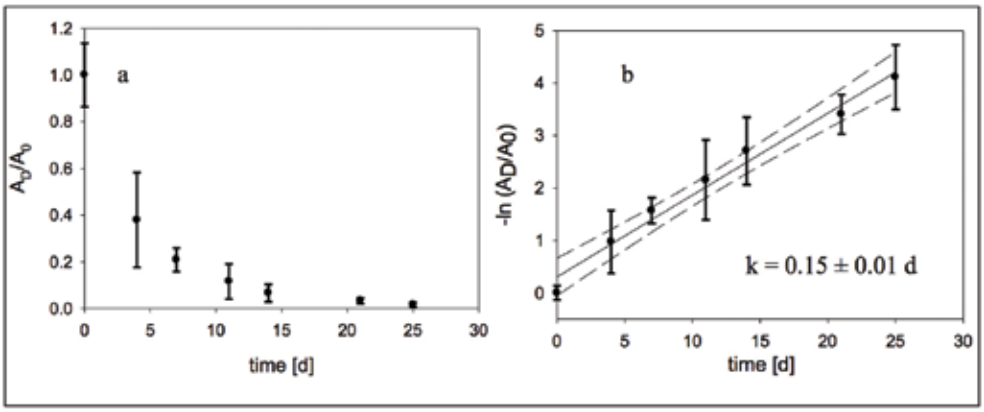


Figure 6. (a) Time-dependent amplification ratio (AD/A0) behavior for encapsulated ssDNA (C4) in acidic solution (pH 2.0). (b) Calculation of the rate constant: The natural decay of the ssDNA was transferred using a half- logarithmical plotting, the slope of the straight line corresponds to the rate constant k of the reaction (dashed lines: 95% confidence band.)

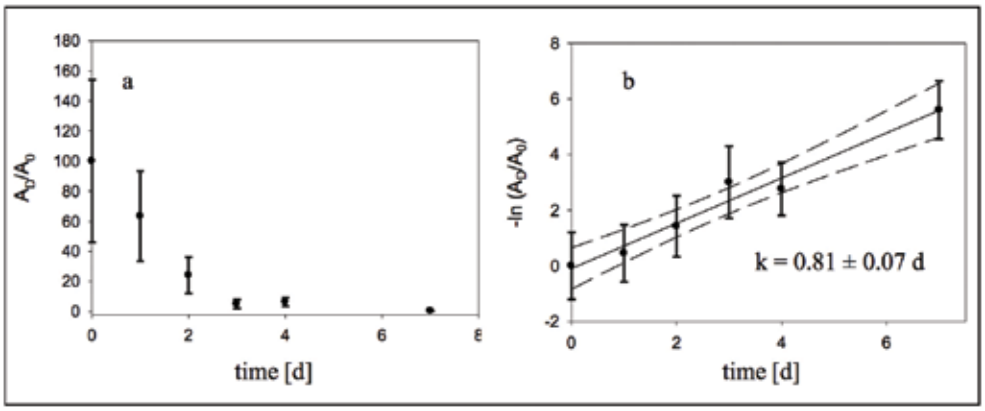


Figure 7. Time-dependent amplification ratio (AD/A0) behavior for encapsulated ssDNA (C4) at 80°C in wet environment (a) and calculation of the rate constant (b) (dashed lines: 95% confidence band.)

submitted to an established alternating climate test and a test of light fastness. During the chosen alternating climate test, the leathers are subjected to several changes between humid cycles (90% air humidity) and hot cycles (120°C), because high temperatures and humidity are two main reasons for DNA damage. Such a climate test is called "desert test". Although it displays extremely high temperatures, the test was chosen, because under definite circumstances leathers have to face such temperatures, e.g. in automotive interiors the leather of the dash board or seats is heated during extensive sunlight irradiation. The test of light fastness simulates sunlight, which also causes DNA damage. For both tests, the ssDNA was isolated from the base coat of the leathers before and after conducting the test procedure and the DNA damage was measured with qPCR (Figure 4).

ssDNA could be isolated from the base coat of the leather without and with finishing layer on top (Figure 4). This ssDNA concentration was used as starting concentration to evaluate the DNA damage caused by the two standard test procedures.

After the alternate climate test, non-encapsulated ssDNA is no longer detectable. The extreme conditions simulated by the test, destroy unprotected ssDNA and the stored information is irrecoverably lost. After 192 h of simulated sunlight radiation, the amount of ssDNA is decreased by about four fifth whether the coated leathers were finished with topcoat or not. These results show that a protection of ssDNA is essential in order to use it as leather marking system. A special requirement is needed for the stabilization of ssDNA against high temperatures. Therefore, the ssDNA was attached to hydroxyapatite.

DNA Stability of the Optimized Marking System at pH 2.0 and 80°C (laboratory scale tests)

The optimized marking system used in this study consists of 50% PS-DVB-microcapsules containing ssDNA attached to hydroxyapatite. The marking system was checked for its ability to protect ssDNA against pH 2.0 (threshold in tanning) and against high temperatures in combination with elevated humidity (e.g. drying) in laboratory scale tests. The SEM-picture of sample **C4** (Figure 5) shows globular capsules, indicating that an encapsulation of a hard core (hydroxyapatite) is possible. The synthesized microcapsules showed diameters between 1 and 3.5 µm.

The ssDNA decay over time in sample **C4** at pH = 2.0 is shown in Figure 6. It displays an apparent rate constant of $k = 0.15 \pm 0.01\ d^{-1}$ and an apparent half-life of $t_{1/2} = 4.62 \pm 0.62$ d. Non-encapsulated ssDNA has an apparent half-life of 0.40 ± 0.10 d in acidic TSB of pH 2.0[9]. Therefore, the capsule protects the ssDNA in acidic solution against hydrolytic attack and prolongs the survival of the DNA. However, ssDNA is still degraded despite the protection by the capsule. This indicates that the PS-DVB microcapsules are not completely waterproof and the ssDNA is exposed to hydrolytic attack in the inner core caused by permeating solution.

The calculated half-life of the ssDNA in **C4** at pH = 2.0 is in the same range of the half-life measured for PS-DVB microcapsules without the addition of hydroxyapatite.[9] This finding shows that the half-life of encapsulated ssDNA can be reproducibly measured with the chosen method independently of the consistency of the core. On the other hand, it also indicates that attaching ssDNA to hydroxyapatite (**C4**) does not improve ssDNA stability at pH = 2.0[8]. Therefore, only the capsule is responsible for the protection of DNA in acidic pH solution.

The ssDNA decay for sample **C4** was also investigated at 80°C in wet environment. The After exposing **C4** to 80°C, DNA detection was difficult because the detected concentrations deviated extremely. These problems do not occur during the exposition to low pH or during the stability assay with ssDNA and hydroxyapatite in hot water.[9] Regarding these difficulties; two stability assays with different time intervals (3 and 7 days) were conducted and combined to one amplification curve (Figure 7). Thus, six measured data points in duplets were available for 0, 1, 2, and 3 days. In addition, the level of significance was raised from 5% to 20% ($\alpha = 0.2$) to eliminate more values.

Nevertheless, high standard deviations and ssDNA contents that were higher than the starting ssDNA amount, were still measured. In a previous study, we have shown that abasic sites influence the polymerase progression in qPCR. This influence leads to high standard deviations of the mean amplification ratio.[9] We also showed that during a time interval of 21 days at acidic pH, no abasic sites influence the measurements.[8] Regarding the high standard deviations and the smaller time intervals of 7 and 3 days, abasic sites seem to play an important role during the heat experiment. Because the ssDNA is attached to hydroxyapatite, it can be concluded that the transformation from an abasic site (not detectable in qPCR) to strand breaks (detectable in qPCR) could be retarded by hydroxyapatite. A possible reason for this is its ability to shield the DNA damage points against hydrolysis.

The apparent half-life for **C4** in the laboratory scale test at 80°C was calculated with $t_{1/2} = 0.86 \pm 0.15$ d. Nevertheless, this value must be considered critical, particularly with regard to the difficulties in generating the graphs in Figure 7.

Attachment of PS-DVB Microcapsules to the Collagen-matrix of Leather

We checked whether the optimized leather marking system is able to build a complex bond to the collagen matrix during chrome tanning. Three different microcapsules were synthesized. Sample **C1,** as a reference**,** consisted of 50% PS-DVB microcapsules without functional groups. In contrast, sample **C2** was synthesized with the carboxyl monomer 4-vinylbenzylmalonic acid leading to 5% COOH groups on the surface of the microcapsule (Table 1). Both samples were

fluorescence-labeled with rhodamine side chains in order to locate the microcapsules on the collagen matrix with fluorescence microscopy. As another reference, sample **C4** was produced using neither carboxyl groups nor rhodamine labeling.

Sample **C1, C2** and **C4** were added to commercialized Freiberg Hide Powder during a standard chrome tanning process. Using sample C4, it was investigated whether the side groups on the surface of the PS-DVB microcapsules are necessary. The produced hide powder pellet containing **C4** showed no permanent bonding. Nearly all microcapsules were extracted again by washing [data not shown]. Therefore, a surface modification of the PS-DVB microcapsules is required.

Microcapsule examination of the produced hide powder pellet containing **C2** shows that sample **C2** is fixed to the collagen matrix during tanning (Figure 8b). During chrome tanning, the chrome ions form stable complexes with the collagen side groups. Such complexes can also be formed with the carboxyl groups on the surface of the PS-DVB microcapsules. The hide powder pellets were washed four times after tanning and these wash water fractions were also analyzed using a fluorescence microscope. The first wash water fraction still contained isolated microcapsules. However, their number was so small the large part of **C2** must be attached to the Hide Powder. Hence, the marking system can be fixed into the collagen matrix by carboxyl side groups.

A permanent bonding to the collagen matrix was also possible during tanning with the sample contained **C1**. Again, the first wash water fraction showed only isolated microcapsules. The rhodamine side groups do not undergo a complex fixation to chromium during tanning. However, the cationic part of rhodamine could bind to amino acid side groups of the collagen matrix (e.g. aspartic or glutamic acid), without regard to the type of binding.

Thus, a fixation of the DNA marking system was possible with both carboxyl side groups and rhodamine side groups. Both side groups build stable attachments to the collagen matrix and cannot be washed out after tanning. The results suggest that chrome tanning is not necessary for a marking system with rhodamine side groups. Anyway, this assumption must be analyzed in further experiments.

We also synthesized microcapsules using a 10% COOH-monomer (**C3**) to test the assumption whether a high amount of functional groups on the surface of the microcapsules lead to enhanced bonding during chrome tanning. The microscope analysis showed no visible differences for the quantitative microcapsule distribution in the collagen matrix, indicating that microcapsules synthesized with a 5% COOH monomer (**C2**) are adequate as a leather marking system.

Marking Trials with the Optimized Marking System

Marking trials were conducted using the samples **C4**, **C5** and **C6**. The microcapsules of sample **C6** were synthesized with carboxyl groups on the surface while the microcapsules of sample **C5** features rhodamine side groups. Sample **C4** contained neither carboxyl nor rhodamine side groups and served as control to investigate the effect of both types of side

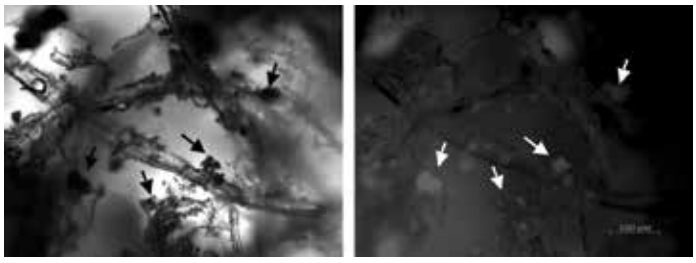


Figure 8. Microscopic images of sample C2 after chrome tanning with hide powder (above: standard mode, below: fluorescence mode.) The arrows show examples of microcapsule accumulation.

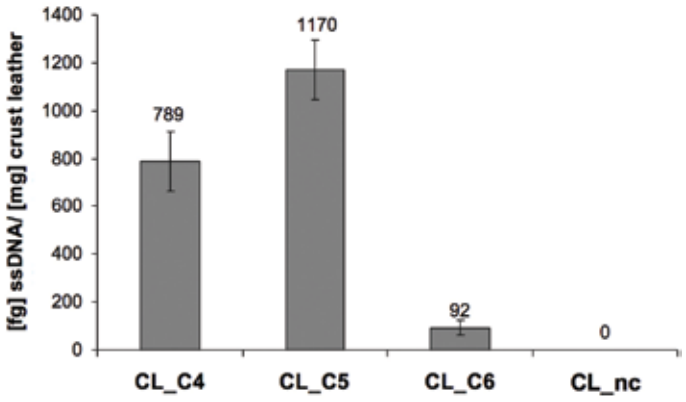


Figure 9. Measured starting ssDNA amount. Every value is an average of three samples of one crust leather measured in duplets.

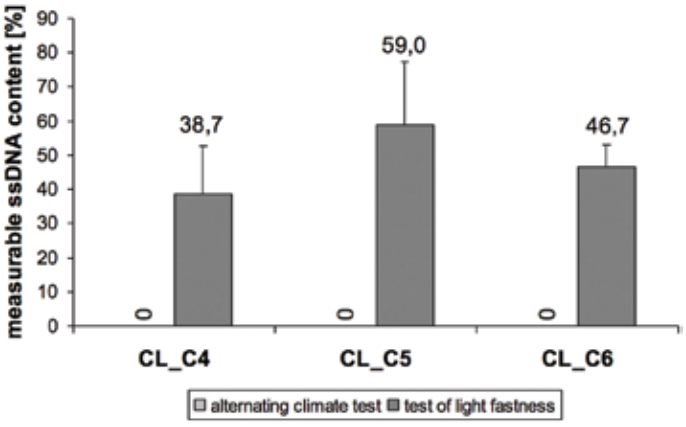


Figure 10. Measured ssDNA content in marked crust leathers after the alternating climate test and the test of light fastness. The measurable ssDNA content refers to 100% starting ssDNA content. CL_C4: crust leather marked with sample C4, CL_C5 and CL_C6 are similarly named. Standard deviations are composed of 6 values.

groups on the fixation of the marking system to the leather collagen matrix. With all three samples, crust leathers were produced: **CL_C4, CL_C5, CL_C6**. A fourth crust leather produced without the addition of the optimized marking system, served as the negative control and was named **CL_nc**. All four crust leathers were subjected to an alternate climate test as well as a test of light fastness. Before the standard test procedures were done, the ssDNA content of the crust leathers was measured and defined as the starting DNA amount (Figure 9). No ssDNA was measurable in **CL_nc**. Interestingly, both crust leathers, **CL_C4** and **CL_C5**, show a higher recovery rate for ssDNA than the crust leather **CL_C6**. The highest amount of starting ssDNA was measured for the sample CL_C15. This finding indicates two things: First, labeling with rhodamine side groups results in a better fixation of the ssDNA-containing microcapsules to the leather matrix than labeling with carboxyl groups. Second, also the sample without side groups (**C4**) is attached to the crust leather. For the crust leather **CL_C4**, a starting ssDNA amount of 789 fg/ [mg] crust leather was measured, which is more than 8 times the amount of ssDNA found in the crust leather **CL_C6** and two third the amount of ssDNA found in the crust leather **CL_C5**. However, the experiment conducted with hide powder showed no obvious binding of a sample without side groups. That means a marking system without side groups cannot build a permanent bond to the collagen matrix. The bonding ability of **C4** in crust leather can be explained by a filling of the leather structure during tanning. During tanning, the marking system is incorporated into the open-pored leather structure together with tanning agents, fats and other additives. Because the open-pored structure is partly closed by these additives, the microcapsules are imbedded into the leather structure. A similar phenomenon is also known from X-Cel leathers (Lanxess, Köln). Such leathers are upgraded by thermally expandable microcapsules. These microcapsules, added during tanning, selectively fill up the loose fiber structure of the leather and several voids, which leads to a more uniform leather structure. In a subsequent step, these microcapsules are expanded and thereby fixated in the leather.[15]

The measurable ssDNA content of the crust leathers after the alternating climate test and the test of light fastness is illustrated in Figure 10. No encapsulated ssDNA could be recovered from the crust leathers exposed to the alternate climate test. Eight days of such extreme conditions like the change between 120°C and 90% air humidity resulted in extreme damage of ssDNA structure,[6] and no ssDNA was amplifiable after the test. However, prior tests on laboratory scale[9] showed a stability enhancement of ssDNA by hydroxyapatite at 80°C and high air humidity. Therefore, the optimized marking system might be adaptable as a marking system for many leather products that are not exposed to these extreme temperatures.

In the test of light fastness, sample **CL_C5** showed the highest recovery rate for measureable ssDNA (Figure 10). It accounts for 59% of the measurable starting ssDNA content. Compared to non-encapsulated ssDNA in crust leather, all samples had recovery rates that were 2 – 3.4 times higher (see values in Figure 4).

Thus, the ssDNA is protected against sunlight radiation by a 50% PS-DVB microcapsule during the test of light fastness. Because different recovery rates are achieved with samples **C4**, **C5** and **C6**, the ssDNA stability cannot only depend on the attachment to hydroxyapatite and the microcapsule shore. Also the side groups can be responsible for stability enhancement. They potentially influence the surface of the microcapsule and change the interaction with UV-light, which is especially significant for rhodamine.

In summary, **C4**, **C5** and **C6** are principally suitable as marking system, because all three capsule variations enhance of ssDNA stability during the test of light fastness.

CONCLUSIONS

For the usage as a leather marking system, the stability of ssDNA must be enhanced at least against acidic pH-values, higher temperatures and ultraviolet radiation as a part of sunlight radiation. ssDNA, which is attached to hydroxyapatite and encapsulated in a 50% PS-DVB microcapsule, achieves these requirements. The results of the laboratory scale test indicate that this optimized marking system shows an enhanced ssDNA stability at pH 2.0 and 80°C. Furthermore, the marking trials with the optimized marking system indicate a protection of ssDNA against sunlight radiation. Therefore, our new marking system based on PS-DVB microcapsules containing ssDNA attached to hydroxyapatite is applicable for leathers with common handling. Unfortunately, stability of the marking system at extreme conditions during the chosen alternate climate test was not achieved.

There seems to be no need to equip the microcapsules with carboxyl groups for an attachment to the collagen leather matrix. A fixation in the leather is possible using carboxyl or rhodamine side groups, probably due to complex bonding. However, an attachment to the leather matrix was also found for the sample without side groups inducing that PS-DVB microcapsules do not need side groups for an attachment. Furthermore, it must be considered that with rhodamine side groups the color of the leather might be influenced. A 50% PS-DVB microcapsule, which contains ssDNA attached to hydroxyapatite and which is equipped with no side groups, is currently the best choice for labeling leather with ssDNA. In conclusion, we successfully showed the approach to install a DNA based leather marking system, which is suitable for supplier and batch tracing. Up to now, the drawback of this marking system is its marginal stability during a special alternate climate test, which reaches temperatures of 120°C and 90% air humidity.

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REFERENCES

1. Gavent, G., Valot, E. XXIX IULTCS Congress, 2007, Intelligent traceability: an innovative technology in the leather industry.
2. Lindahl, T., Nyberg, B.; Rate of depurination of native deoxyribonucleic acid. *Biochemistry* **11**, 3610-3618, 1972.
3. Lindhal, T., Karlström, O.; Heat-induced depyrimidination of deoxyribonucleic acid in neutral solution. *Biochemistry* **12**, 5151-5154, 1973.
4. Beckman, K., Ames, B.; Oxidative decay of DNA. The *Journal of Biological Chemistry* **272**, 19633-19636, 1997.
5. Sinha; R., Häder, D.; UV-induced DNA damage and repair: a review. *Photochemical and Photobiological Science* **1**, 225-236, 2002.
6. Lindahl T. Instability and decay of the primary structure of DNA. *Nature* **362**, 709-715, 1993.
7. Bohrisch, J., Hahn, M., Maedler, A., Stoll, M.; Synthesis and Properties of New Thermosensitive Polymer Systems. *Journal of Applied Polymer Science* **104**, 2122-2129, 2007.
8. Stenzel, S., Bohrisch, J., Meyer, M.; Enhancing synthetic single strand DNA stability at acidic pH by encapsulation for the usage as DNA marking system. *Journal of Applied Polymer Science* **132**, 2015, in press.
9. Stenzel, S., Meyer, M.; Stability of synthetic single-strand DNA as a criterion for the usage as marking system. *Journal of Advanced Biotechnology and Bioengineering* **2**, 30-38, 2014.
10. Yakes, F.M., Chen, Y., van Houten, B.; In: Pfeifer, G., editor. Technologies for Detection of DNA Damage and Mutations. New York: Plenum Press, 169-182, 1996.
11. Trung, N., Francis, M. B.; Practical Synthetic Route to Functionalized Rhodamine Dyes. *Organic Letters* **5**, 3245-3248, 2003.
12. Endo, T., Marnoka, S., Yokozawa, T.; Preparation and polymerization of spiroorthacarbonate containing distyryl structure. *Journal of Polymer Science Part A: Polymer Chemistry* **25**, 2925-2929, 1987.
13. Pat. Appl. WO 01/13179 A1
14. Ruckenstein, E., and Sun, Y.; Polyaniline-containing electrical conductive composite prepared by two inverted emulsion pathways. *Synthetic Metals* **74**, 107–113, 1995.
15. N.N. LANXESS upgrade leather with Levotan X-Cell. In: Leather International 209, No 4778, p. 67, 2007.

SUPPLEMENTARY MATERIALS PROVIDED BY THE AUTHOR

Characteristics of the used synthetic ssDNA
Name: 1/50

sequence (5' to 3'): CGA GAA GCA CTG TGG ATG AAC GCG TTA GAG TAC CTG CTC ACT GTC CTC CT

% GC: 54

Purity: desalted

Primer position: Forward 1-20, Reverse 31-50

qPCR conditions
qPCR was performed in low-profile, 96-well, clear PCR plates sealed with Microseal[å] 'B' Film on the CFX96™ Real-Time-System (all from Bio-Rad, München, Germany). Each qPCR-reaction (20 µl) contained 1x PCR buffer S, 2 mM $MgCl_2$, 100 nM dNTPs, 0.25 U Hot *taq*-DNA-polymerase (all from peqlab), 33 pmol forward and 33 pmol reverse primer (Life Technologies GmbH), and 1x SYBR® Green I (Sigma-Aldrich, Hamburg, Germany). The PCR parameters consisted of an initial denaturation step at 95°C for 3 min, followed by 29 cycles of 95°C denaturation for 10 sec, 55°C annealing for 15 sec and a 72°C elongation for 15 sec, followed by a melt curve from 55°C to 90°C in 0.5 increments.