# A Permanent and Environmental Stable Marking System for Leather -Traceability via Encapsulated DNA

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**Abstract:** Native DNA is unstable under the predominant conditions during the leather production. To establish a new marking system for leather based on DNA, encapsulated DNA was integrated into a conventional basecoat. It was shown that this marking system can be readout from the finished leather. In addition, the stability of the capsules under extreme acidic conditions shows a satisfactory extent for the tanning process, which takes at most four days. Also a protection of the capsule DNA against sunlight radiation could be shown.
**Key words:** encapsulation; DNA; leather; traceability

## 1 Introduction

Traceability and transparency in production processes become more and more important in a global market. For example, the sale of faked leather products in Italy, one of the main leather producing countries in the EU, was estimated at $1.4 billion per year.

However, there is still no suitable long term marking system for leather. At the moment leather is marked with a conventional sticker. But the labelling of stickers does not satisfy the high requirements to a 100% secure marking system. The great advantage of a DNA-based marking system would be that the leather can be marked area-wide. Furthermore with DNA huge amounts of information can be stored for one product. For example, as much as $4^{20}$ combinations for a variety of information are available with only 20 base pairs (bp). Furthermore, with DNA the leather can be already labelled during its production process whereas the sticker would mark only the finished product.

But for integration of DNA into unfinished leather, the DNA should be stable against certain environmental effects during the leather production process, such as acidic and alkaline pH, elevated temperatures or oxidizing, reducing and chelating agents, like $H_2O_2$, $Na_2S$ or Nitrilotriacid (NTA). Native DNA is usually damaged under these conditions. The result is the formation of abasic sites [1, 2], which is accompanied by a loss of information and strand breakage [3], or the formation of photoproducts and other lesions [4]. One possibility for protection of synthetic DNA against such environmental influences is its encapsulation.

There are other attempts for designing new marking systems, e.g. the labelling with powder with magnetic resonance or microspheres encapsulating either fluorophores or synthetic DNA [5]. These new approaches with synthetic DNA were only tested by the integration of these new technologies during the leather finishing.

The aim of this project is the encapsulation of DNA, in order to stabilize the system against all environmental effects during the steps of the leather production process, thus enabling for encapsulated DNA to be integrated during the tanning or the wet end procedure, as well as during the finishing process. Furthermore, research work aims on giving proof of the durable and long-term stable characteristics of the encapsulated DNA.

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## 2 Experimental

### *2.1 Materials*

Plasmid pBR322 was purchased from fermentas® (Germany), all oligonucleotides and primers for PCR were obtained from invitrogen® (Karlsruhe, Germany). PCR reagents and agarose for agarose gel electrophoresis were supplied by peqLab® (Erlangen, Germany), all other chemical substances were supplied either by Carl Roth® (Karlsruhe, Germany), Sigma-Aldrich® (Seelze, Germany) or Merck® (Darmstadt, Germany).

### *2.2 Preliminary Test with Native Plasmid DNA*

A preliminary test on DNA stability was accomplished with plasmid DNA. Therefor 0,5 µg of the plasmid pBR322 was incubated for one hour under UV-light (254 nm, 25 W), at 80°C in Theorell-Stenhagen-buffer (33 mM citric acid/ 33 mM phosphoric acid/ 57 mM boric acid/ 34.4 mM NaOH / pH adjusted with 0.1 M HCl) pH 7.0, in Theorell-Stenhagen-buffer pH 7.0 supplemented with 30 µM $H_2O_2$ and 10 mM $C_4H_6CuO_4$ [6] and in Theorell-Stenhagen-buffer of pH 2.0, pH 7.0 and pH 12.0. The complete plasmid preparation was resolved on a 1% vertical agarose gel by electrophoresis in TAE buffer (40 mM TRIS, pH 8 / 20 mM acetic acid / 1 mM Na-EDTA, pH 8) at 70 V for one hour.

### *2.3 Encapsulated Oligonucleotides*

Three single-strand (ss) oligonucleotides (1/50, 2/60, 3/70) of different length and different primer binding sites were designed. The encapsulated DNA consisted of one of these three oligonucleotides, incorporated into a gelatine/ water- or polyacrylamide/water matrix, which was surrounded by crosslinked polystyrene. The capsules were stored in isoparaffine. The detailed composition of the encapsulated DNA is shown in Tab. 1, their principal composition is illustrated in Fig. 1.

**Tab. 1 Composition of the encapsulated samples**

| Sample | DNA | Crosslinking of polyacrylamide | Gelatine matrix | Crosslinking of the polystyrene shell | Temperature of polymerisation |
|---|---|---|---|---|---|
| BCD36 | 3/70 | - | 3% | high | 45°C |
| BCD38 | 2/60 | - | 6% | high | 45°C |
| BCD40 | 1/50 | high | - | medium | 45°C |
| BCD66 | 2/60 | - | 6% | highest | 45°C |

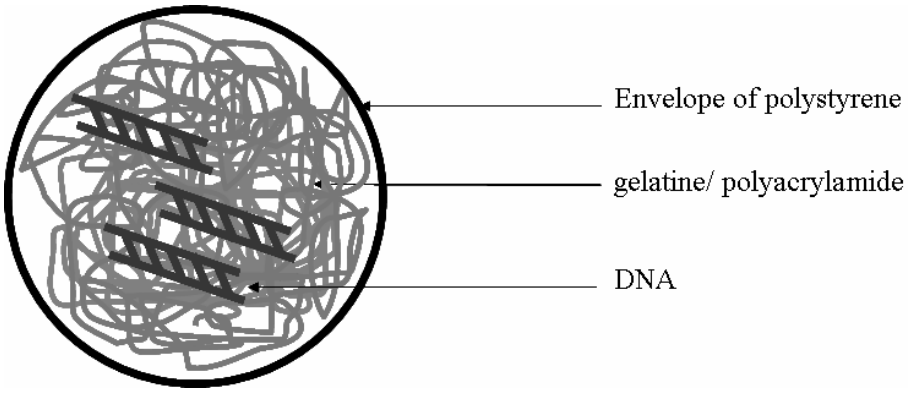


**Fig. 1 Composition of a polystyrene capsule**

### *2.4 DNA Isolation and Detection of DNA*

The DNA was released from the polystyrene capsules by mechanical extraction. The capsules were shock-frozen with liquid nitrogen. After addition of glass beads the capsules were destroyed with a swing mill. Afterwards the DNA was isolated with isopropanol. Therefore, sodium acetate at an end concentration of 0.3 M was added. The DNA was precipitated with 0.7 percent by volume of isopropanol. After centrifugation at 10000 rpm for 10 min the pellet was washed with 70% aqueous ethanol and air dried. The dried pellet was mixed with 30 µl $ddH_2O$.

Each of the three single-strand oligonucleotides was detected by amplifying it in a Polymerase Chain Reaction (PCR) with specific primer pairs for every oligonucleotide. With the program BLAST[7] it was ensured, that no synthetic primer binds to the DNA of higher animals such as cattle, sheep, goats, pigs or humans.

The PCR parameters for all qualitative and quantitative DNA detections 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.

For a qualitative DNA detection the amplified DNA was screened on a 3% vertical agarose gel by electrophoresis in TAE buffer.

The quantitative detection was performed with the CFX96™ Real-Time system (BIO-RAD) in combination with SYBR® Green as dye. To ensure a specific PCR-product, a melt curve from 55°C to 90°C in 0.5 increments was arranged after every PCR run.

### *2.5 Stability Assays*

In order to analyze which environmental influences destabilize the marking system, the capsules were exposed under different conditions (up to now these are extreme acidic conditions simulating the tanning process and sunlight radiation). If a specific PCR-product of the right length can be obtained after the treatments, the marking system is expected to be stable enough to resist the influences during the leather producing process and the environmental effects on a finished leather. All publications about DNA damage illustrate results about specific DNA lesions. In contrary the aim of this work is to determine conditions which ensure at least one remaining stable DNA molecule, from which any stored information of the marking system can be read out.

For the stability experiments under acidic conditions the encapsulated DNA was treated with a Theorell-Stenhagen-buffer simulating pH values of pH=2.0 and pH=7.0, respectively. Aliquot samples of 25 mg were stored in 100 µl buffer of the different pH values for a time period of 30 days at room temperature and in the dark. After incubation the acidic samples were neutralised before DNA isolation and quantitative DNA detection.

For the stability experiments under sunlight exposure a Suntest CPS + system was used. 10 mg of the encapsulated DNA were exposed to a total dose of 360 000 $kJ/m^2$ for 200 h. The exposure was followed by DNA isolation from the capsules and a qualitative detection of the DNA.

All stability assays were also carried out with synthetic, non encapsulated DNA to ensure a comparison of the stability of encapsulated and non encapsulated DNA.

## 3 Results and discussion

### *3.1 Recovery of Encapsulated DNA from the Finishing Coat*

The designed polystyrene-capsules which encapsulate oligonucleotides are displayed in Fig. 2. Their diameter ranges between 1 to 5 µm, which is comparable to that of bacteria. The bacterial diameter ranges

between 0.2 µm for nanobacteria and 750 µm for e.g. *Thiomargarita namibiensis* [8]. The capsules possess a nearly ideal globular structure.

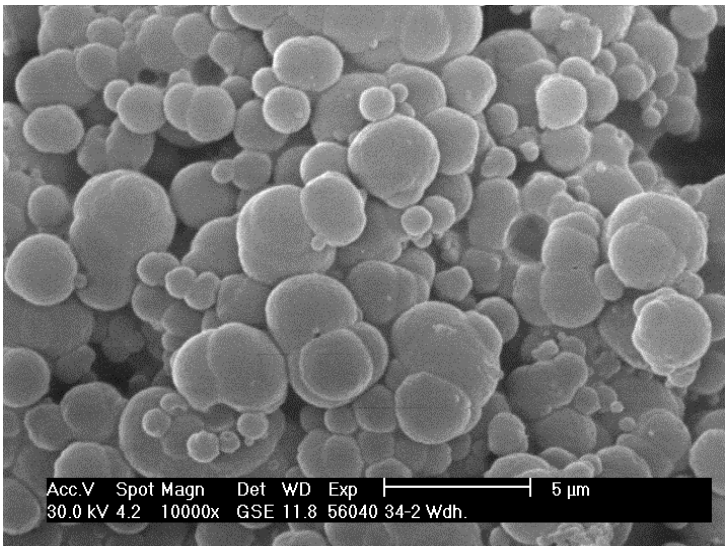


**Fig. 2 Polystyrene capsules with integrated ss DNA (detected by SEM)**

It was possible to integrate the polystyrene capsules into a conventional leather basecoat and impregnate leather with this marked basecoat. Topcoat and finishing coat were printed over the basecoat. A profile of the marked leather is shown in Fig. 3.

For recovering of the oligonucleotides from the inside of these polystyrene capsules located in the finished leather the finishing coat was removed from the substrate and milled followed by mechanical extraction of the DNA in combination with a DNA isolation. A qualitative detection of the specific PCR-product was successful. Thus the polystyrene-DNA capsules are a potential marking system for leather, because integration in leather and recovery is possible.

### *3.2 Plasmid Stability*

To explore the influence of different environmental effects on the stability of DNA, preliminary tests were accomplished with the native, non-encapsulated plasmid pBR322. The advantage of this circular, double stranded (ds) DNA molecule over a linear single stranded (ss) DNA molecule (which shall be used as the marking system) is the more facile detection of single-strand-breaks (circular conformation) and/ or double-strand-breaks (linear conformation) on agarose gels [6].

Fig. 4 illustrates the different results for the tested environmental influences. Lane 5 demonstrates the positive control in the supercoiled formation. DNA lesions are caused by high temperatures (lane 6), oxidizing agents such as $H_2O_2$ (lane 3), UV-light (lane 8) and acidic and alkaline conditions in combination with raised temperatures (lane 9 and 10). Incubation in acidic buffer does not lead to visible DNA lesions, because apurinic sites occur under acidic conditions, which lead to strand breakage only after a few days [9].

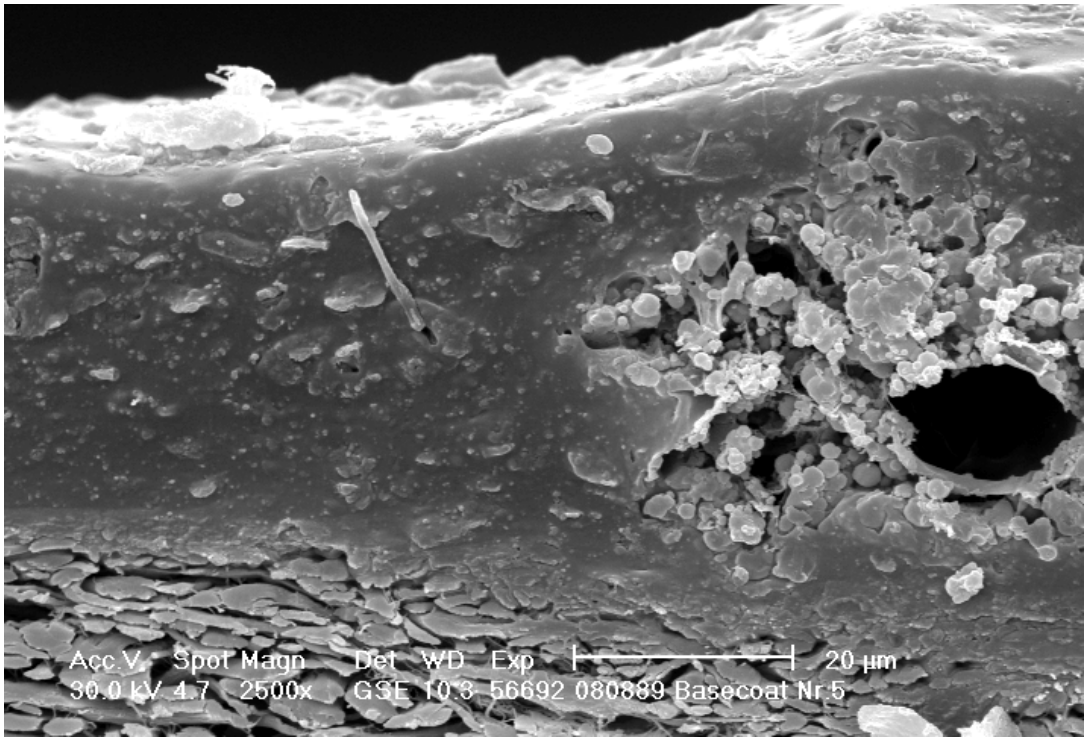


**Fig. 3 Profile from marked leather. (detected by SEM)**

(bottom area: leather structure, upper area: finishing coat with the integrated polystyrene capsules which encapsulate a defined amount of oligonucleotides)

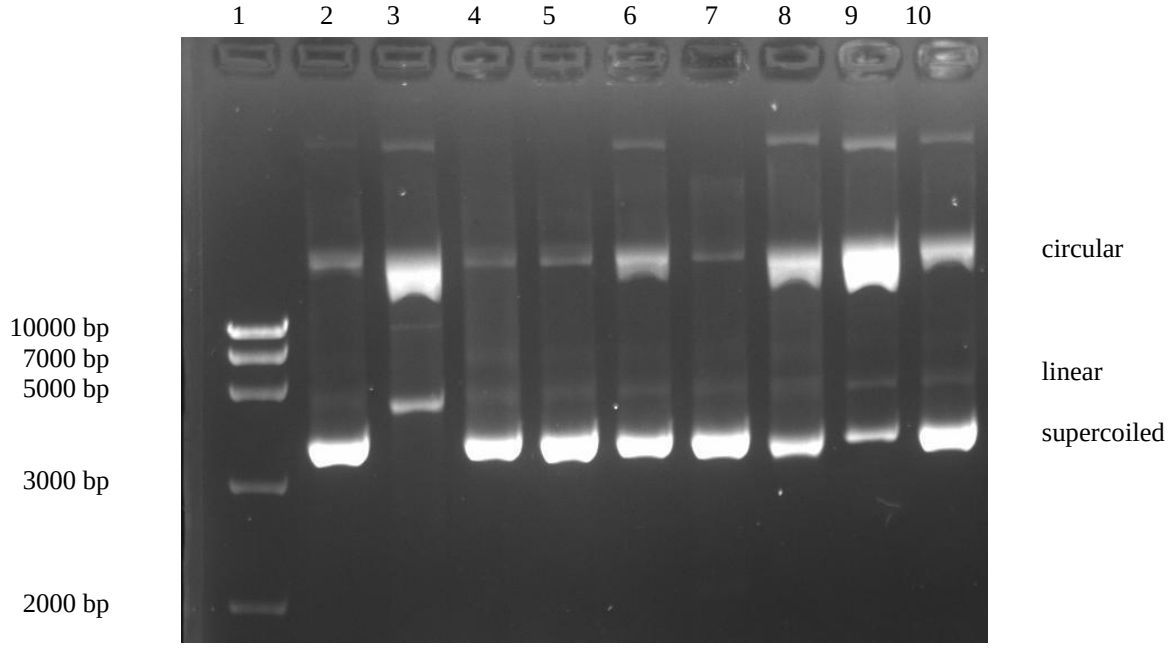


**Fig. 4 Influences from different environmental effects on the stability of plasmid pBR322.**

Lanes: (1) MassRuler$^{TM}$ Express Forward DNA Ladder Mix (ready to use); (2) 10 µM $H_2O_2$ and 10 µM $Cu^{2+}$; (3) 10µM$H_2O_2$ and 10 mM $Cu^{2+}$; (4) pH= 2,0; (5) pH= 7,0; (6) 80°C; (7) pH= 12,0; (8) UV-light; (9) pH= 2,0 and 80°C; (10) pH= 7,0 and 80°C.

### *3.3 Stability of Encapsulated DNA under Sunlight Radiation*

The finished leather is exposed to sunlight under normal usage. Therefore, the stability of sample BCD38 was tested under sunlight exposure. After 8 days enough DNA remained stable to be amplified by PCR. The obtained PCR-product had also the right length and sequence for the primer binding site. In contrast, the same amount of non encapsulated synthetic DNA is not stable under sunlight exposure (data not shown). So the stability of synthetic ss DNA under sunlight radiation is increased by encapsulation with polystyrene.

### *3.4 Stability of Encapsulated DNA under Acidic Conditions*

Acidic conditions are predominant during tanning in the leather producing process as well as in the final product. As described elsewhere, such conditions lead to lesions in the DNA molecule which can block the amplifying enzyme in the PCR, the taq-polymerase [9,10].

Therefore, the stability of the encapsulated DNA was checked for pH 2.0 and 7.0, respectively. The results for sample BCD40 are summarized in Fig. 5.

At pH 7.0 depending on the time all concentration values disperse about a mean concentration. The DNA molecules in the polystyrene capsules are distributed inhomogeneously, therefore, but a decrease of the DNA concentration is not observed.

In contrast, under acidic conditions of pH 2.0 a decrease of the DNA concentration for BCD40 was detected over a period of 29 days. Abasic sites are generated in native, non encapsulated DNA under acidic conditions, which lead to strand breakage after a few days [2,3,9]. This rapid decrease of the DNA concentration suggests that the capsules are not compact enough against protons. Nevertheless, after an incubation period of 29 d at pH=2.0 the encapsulated oligonucleotides are still detectable. The results for BCD36, BCD38 and BCD66 are comparable to the obtained data for BCD40 (data not shown).

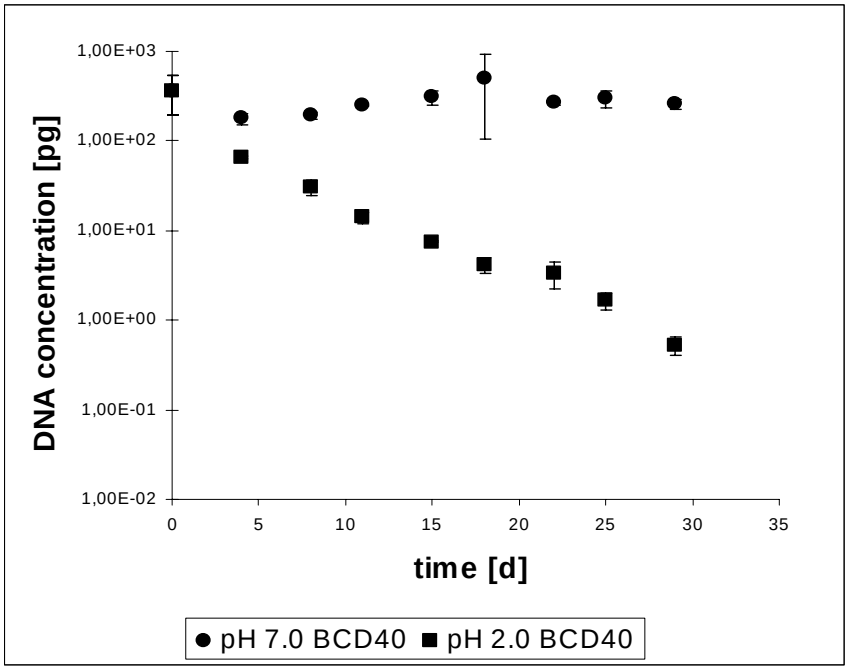


**Fig. 5 Stability of BCD40 under acidic conditions**
(logarithmic scaling, standard deviation refer to a threefold measurement of each data)

Considering a tanning process of 3 days and its low pH values the stability of the capsules under acidic conditions is satisfactory, because in a smaller time period of 8 to 10 days the DNA amount decreased just about 10-fold in an acidic environment (Fig. 6). By contrast, for synthetic DNA, which is not protected via a polystyrene capsule, only 0,63% of the initial DNA amount were detectable after an acidic treatment over 7 days. Thus it is shown, that the stability of DNA in an acidic environment can be increased by integrating the DNA into a polystyrene shell.

Also in a neutral environment the stability of encapsulated DNA is higher than for non encapsulated, synthetic DNA. After a treatment over 7 to 10 days only 40% of the initial amount of synthetic DNA could be measured, whereas for the encapsulated DNA 70% (BCD40) up to 100% (BDC36, CD38 and BCD66) could be measured. That means: Nearly no DNA molecule is damaged under neutral conditions by the protection of a polystyrene capsule at room temperature.

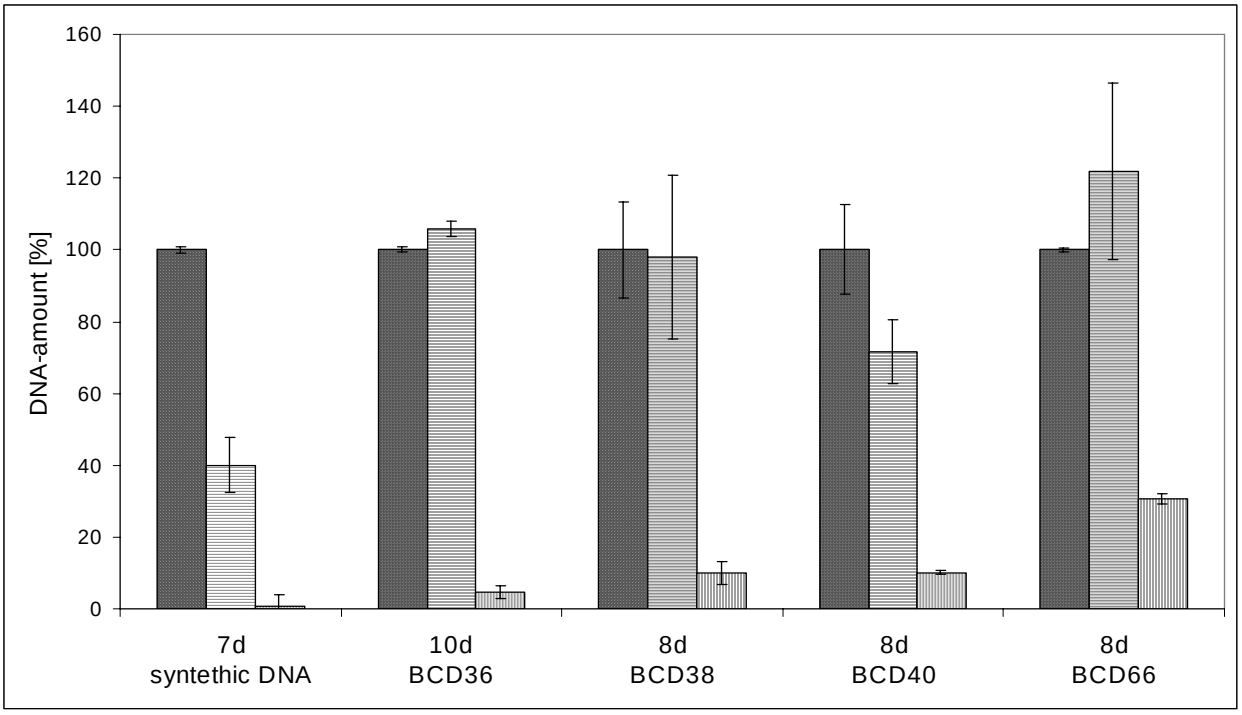


**Fig. 6 Stability of oligonucleotides encapsulated in various polystyrene capsules under neutral and acidic conditions in contrast to synthetic oligonucleotides**

(standard deviation refer to a threefold measurement of each data)

Initial DNA amount, measured at pH7

DNA amount after treatment with a buffer of pH=7.0 over 7 to 10 days at room temperature

DNA amount after treatment with a buffer of pH=2.0 over 7 to 10 days at room temperature

However, it is necessary to mention the fact, that finished leather has a slightly acidic pH between pH 4 to 5. A long-term stability of the encapsulated DNA against such slightly acidic conditions still has to be demonstrated.

## 4 Conclusions

Up to now the polystyrene capsules protect the encapsulated DNA against sunlight radiation. The DNA stability is increased under acidic and neutral environments by a polystyrene capsule over a low time period. To improve the stability under slight acidic conditions over a longer time period, the

structure of the capsule and the inner matrix of the capsules have to be improved, e.g. by embedding the DNA in the inside of the shell in stabilisation solutions or matrices such as spermine [6] or hydroxyapatite. The adsorption of DNA to hydroxyapatite is known to reduce depurination [9]. This adsorption could be jointly responsible for the stability of ancient DNA [9], so that studies from the genome of Egyptian mummies [11], mammoths [12] or Neanderthals [13] are possible.

After an optimization of the capsules the encapsulated DNA should brave the environmental influences in the leather producing process, so that encapsulated DNA becomes interesting to the leather industry as a new application for a 100% secure marking system.

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**References**

[1] T. Lindahl; O. Karlstrom. Biochemistry, 1973, 12: 5151-5154.
[2] J. Zoltewicz; D. Clark; T. Sharpless; G. Grahe. Journal of the American Chemical Society, 1970, 92: 1741-1750.
[3] T. Lindahl; A. Andersson. Biochemistry, 1972, 11: 3618-3623.
[4] R. Sinha; D. Häder. Photochemical & Photobiological Sciences, 2002, 1: 225-236.
[5] G. Gavent; E. Valot. XXIX IULTCS Congress, 2007, Intelligent traceability: an innovative technology in the leather industry.
[6] H. Ha; J. Yager; P. Woster, R. Casero. Biochemical and Biophysical Research Communications, 1998, 244: 298-303.
[7] S. Altschul; T. Madden; A. Schaffer; J. Zhang; Z. Zhang; W. Miller; D. Lipman. Nucleic Acids Research, 1999, programs 25: 3389-3402.
[8] H. Schulz; B. Jørgensen. Annual Review of Microbiology, 2001, 55: 105-137.
[9] T. Lindahl. Nature, 1993, 362: 709-715.
[10] B. Van Houten; D. Chandrasekhar; W. Huang; E. Katz. In Technologies for Detection of DNA Damage and Mutations, New York, ed. G.P. Pfeifer, 1996: 169-182.
[11] S. Paäbo. Genome Research, 1991, 1: 107-110.
[12] J. Krause; P. Dear ; J. Pollack; M. Slatkin; H. Spriggs; I. Barnes; A. Lister; I. Ebersberger; S. Paeaebo; M. Hofreiter. Nature, 2006, 439: 724-727.
[13] M. Krings; A. Stone; R. Schmitz; H. Krainitzki; M. Stoneking; S. Paeaebo. Cell, 1997, 90: 19-30.