Enhancing ssDNA stability at acidic pH by encapsulation for the usage as DNA marking system

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ABSTRACT: The usage of synthetic single-strand DNA (ssDNA) as an invisible barcode is critical for products, which are exposed to DNA damaging influences during their production or handling (ultraviolet light, acidic conditions, and high temperatures). Encapsulation may protect ssDNA against hydrolytic attack under acidic conditions. This assumption was proved by embedding ssDNA into a spherical hydrogel matrix of polyacrylamide and coating it with a crosslinked polystyrene shell. After acidic treatment of these capsules, the ssDNA stability was measured by detecting the amplification ratio over time with quantitative real-time polymerase chain reaction and calculating the apparent rate constant and apparent half-life. The results of the quantitative detection of ssDNA damage demonstrated that enhancing ssDNA stability by encapsulation with crosslinked polystyrene is possible. Such a potential application may be used in all fields of traceability and of combating counterfeiting, where protection of DNA against environmental influences is needed, for example, as safe marking system for paper, biomaterials, textiles, or leather. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41754.

INTRODUCTION

The use of single-strand DNA (ssDNA) as a marking system is not a new idea due to its huge code diversity and the possibility of comprehensive invisible labeling, which allows also marking of liquids, bulk goods, and whole areas of flat materials. In the field of food industry, traceability via internal naturally occurring DNA is already established.[1–3] The ubiquitous presence of DNA in food is a great advantage which enables the determination of product origin just by analysis of a small piece of food. Also in groundwater flow studies, DNA tracers consisting of synthetic single-stranded oligonucleotides are successfully applied.[4]

Principally, DNA can outlast huge time intervals. This is demonstrated by the effectual determination of a mitochondrial DNA sequence from a neandertal-type specimen[5] or of DNA fragments from an Egyptian mummy.[6] But such an enormous DNA stability requires specific circumstances, such as, cool temperatures, dry environments, and neutral pH.[7]

Elevated temperatures, acidic environments, or ultraviolet (UV) radiation damage DNA molecules. A DNA-based marking system exposed to these environmental impacts would lose its stored information due to modified bases, abasic sites, and strand breakage (for review see Ref. 7). For this reason DNA tracers are not suitable for flow water studies in mine waters.[4] Similarly, an invisible DNA label is a great challenge for products, which are exposed to acidic conditions during their production, like paper (e.g., bank notes), biomaterials (e.g., medical devices from collagen), textiles, or leather. Encapsulation of marking DNA could be one solution for this complicacy. In the past, Bohrisch *et al.* stabilized hydrogels with a crosslinked polystyrene casing.[8] We adopted this idea by embedding ssDNA into a hydrogel sphere and coated it with a shell of crosslinked polystyrene. By use of these polystyrene–hydrogel microcapsules we aimed to enhance ssDNA stability against acidic environments for the use as a marking system.

We measured the stability of this encapsulated DNA at pH = 2.0 as a realistic example for acidic conditions, for example, in the leather industry. The induced DNA damage was detected by amplification of ssDNA in a quantitative real-time polymerase chain reaction (qPCR) and calculation of the amplification ratio was done between the treated and untreated encapsulated ssDNA. This method is based on the premise, that many DNA lesions have the ability to block or at least obstruct the DNA

Table I. Composition of the Encapsulated ssDNA Samples (Ingredients before Encapsulation)

Sample	Amount of DNA	Oil phase composition (g)		Proportion of DVB in the polystyrene shell
		Styrene	DVB	
C1	20 µg	7.25	2.41	25%
C2	20 µg	5.75	3.83	40%
C3	20 µg	4.83	4.83	50%
C4	20 µg	2.41	7.25	75%
C5	20 µg	0	9.66	100%
C6	–	5.75	3.83	40%

The proportion of DVB is characteristic for the rate of shell linkage, C6 is the negative control without ssDNA.

polymerase.[9–11] The amplification ratio strongly depends on the amount of amplifiable DNA. Because the total DNA damage accumulates over time, the time-dependent amplification ratio decreases and the associated rate constant and half-life are calculated from that decrease. These calculated constants represent the accumulation velocity of non-amplifiable ssDNA damage and constitute apparent constants, because they only represent the velocity of DNA damage which is detectable with qPCR.[12]

The aim of our investigation was, therefore, the use of hydrogel microcapsules to enhance ssDNA stability against acidic environments for the usage as a marking system.

EXPERIMENTAL

Materials

All solvents except Isopar (high boiling mixture of iso paraffins; ICI) and methylenebisacrylamide were supplied by Sigma-Aldrich and used as received. Span 80, Tween 85, ethylenediamine tetraacetic acid (EDTA), styrene, divinylbenzene (DVB), acrylamide, and N,N′-Hypermer 2296 (ICI), 2,2′-azobis-(2,4-dimethylvaleronitrile) (V-65, Wako), and 2,2′-azobis-(N,N′-dimethyleneisobutyramidine) dihydrochloride (VA-044, Wako) were of commercial grade and used as provided. Synthetic ssDNA (1/50) and the associated primers for PCR analysis were designed manually and were obtained from Life Technologies GmbH (Darmstadt, Germany). For sequences of 1/50 and the used primers refer article by Stenzel and Meyer.[12]

Synthesis

Six different variants of microcapsules (C1–C6, Table I) were synthesized in two steps. Step 1: Inverse emulsion polymerization of the DNA containing hydrogel. The 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), ssDNA 1/50 (20 µg), and water (19.34 g) and organic phase containing Isopar M (6.51 g), Span 80 (0.55 g), Tween 85 (1.10 g), and Hypermer 2296 (0.07 g) were mixed for 1 min at 3000 rpm. The mixture was transferred into a nitrogen flushed lab reactor at 45°C, 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. After 24 h the polymerization was stopped. Step 2: Microencapsulation of C1–C6: A solution of Isopar M (37.19 g), Span 85 (2.97 g), Tween 61 (1.61 g) styrene, and DVB was mixed for 1 min at 3000 rpm. The crosslinking rate of the polystyrene shell was modified by varying the proportion of the crosslinking agent

DVB (for proportions see Table I). Afterwards the gel particles of step 1 (32 g) were added, and the mixture was again stirred for 1 min at 3000 rpm. It 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 added quickly. After 4 and 8 h, further portions of V-65 were added. After 24 h the polymerization was stopped. In order to remove impurities and most of the surfactants, the polystyrene-co-divenylbenzene (PS-DVB) microcapsules were allowed to settle and washed three times with hexane.

Stability Assays/Sample Treatment

Aliquoted samples of encapsulated ssDNA (25 mg each, C1–C5) were incubated in 200 µL Theorell–Stenhagen buffer (TSB, pH 2.0; 33 mM citric 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, 10, 14, 21, and 28 days for C2–C5; 0, 2, 3, 7, 14, and 21 days for C1), three aliquots of C1–C5 were incubated in the dark and at room temperature. After sample treatment the acidic samples were neutralized with TSB (without HCl) and digested. The released ssDNA was analyzed by qPCR for total ssDNA damage.

Digestion of PS-DVB Microcapsules and DNA Release

ssDNA was released from PS-DVB microcapsules by mechanical destruction. The samples were shock-frozen in liquid nitrogen without removal of neutralized TSB and vortexed in the presence of glass beads (Ø 0.1–0.25 mm) and 300 µL of sterile bidest water. The obtained ssDNA solution was directly used to analyze the ssDNA damage by qPCR.

qPCR and Analysis of qPCR-Data/Statistics

The used qPCR assay, the analysis of the qPCR data and the statistics were adopted from a previous study.[12] In short, the total DNA damage was detected by amplifying the treated and non-treated DNA with qPCR based on SYBR Green I dye detection. 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[11] 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, statistic analysis was carried out with Dixon's Q-test ($n = 3$; $\alpha = 0.05$). The amplification ratio

Table II. Inhibition Control with Digestion Solution and Sample C6

c_q for employed DNA concentrations							
DNA	100 pg	10 pg	1 pg	10 fg	1 fg	R^2	E
Intra	5.54 ± 0.02	8.99 ± 0.04	12.63 ± 0.14	19.69 ± 0.28	22.78 ± 0.06	0.999	93.9%
Inter	5.35 ± 0.23	8.78 ± 0.25	12.47 ± 0.20	19.65 ± 0.17	22.65 ± 0.17	0.999	93.1%
Absolute	–	8.74 ± 0.22	–	–	22.73 ± 0.18	0.999	94.9%

Abbreviations: intra, intra assay variation, measured with one sample in duplets; inter, inter assay variation, measured with two samples in duplets; absolute, absolute quantification, correlation with standards.

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 apparent rate constant k was calculated by linear regression using SigmaPlot 11.0. The apparent half-life $t_{1/2}$ was determined with the equation $k = \ln2/t_{1/2}$.

Detection of Abasic Sites and Inhibition Assays

For abasic site detection the extracted damaged ssDNA of **C1** was cleaved with Endonuclease V, *T. maritima* (fermentas, St. Leon-Rot, Germany). The digestion assay was performed as follows: 1 μL sample, 1 U enzyme, 65°C, 30 min in 1× appropriate reaction buffer. Digested samples were analyzed for total damage in qPCR.

The influence of the digestion solution and TSB on qPCR was checked with sample **C6** (see Table I). The microcapsules were either destroyed in the presence of water (influence of the digestion solution) or in a mixture of TSB in water in the same ratio as used in the stability assays under acidic conditions (influence of TSB). Both digestion solutions were mixed with ssDNA of known concentration (100 pg/μL, 10 pg/μL, 1 pg/μL, 10 fg/μL, 1 fg/μL, 0.1 fg/μL). Assay variation was checked by generating standard curves of the c_q-values of **C6** against the logarithmic concentration for intra- and interassay variation. The measured absolute DNA concentration was compared with the employed standards (10 pg/μL, 10 fg/μL, 1 fg/μL, 100 ag/μL, 10 ag/μL) (see Tables II and III, absolute) by generating a standard curve with all c_q-values of both samples and standards.

Scanning Electron Microscopy (SEM)

ssDNA containing PS-DVB microcapsules were imaged with SEM on a Philips XL30 ESEM.

RESULTS AND DISCUSSION

Composition of Encapsulated ssDNA

ssDNA was embedded in an aqueous hydrogel matrix which consisted of 4.7% crosslinked polyacrylamide in water. The inverse emulsion polymerization was performed using acrylamide, the crosslinking agent methylenebisacrylamide and water soluble initiator VA044. The gel particles were stabilized in the solvent Isopar M using a mixture of surfactants of the sorbitan type (hydrophilic–lipophilic balance [HLB] = 8.6). The DNA containing hydrogel particles were used without further purification. They were covered with a crosslinked polystyrene shell by precipitation and polymerization of styrene and DVB. This shell protects the core against mechanical impact and partly prevents diffusion exchange of solvents. A simple procedure to prepare hydrophilic core/hydrophobic shell particles via inverted emulsions was first proposed by Li and Ruckenstein.[13] Following that, the resulting inverse emulsion of step 1 was mixed with an Isopar phase containing the styrene monomers, a mixture of stabilizers (HLB ∼ 4.5) and an oil soluble initiator. Due to the insolubility of crosslinked 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. The polystyrene microcapsule acts as a physical barrier for the ssDNA against external influences such as larger molecules. The crosslinking degree of the polystyrene shell was regulated from medium to high by adjusting the amount of admixed crosslinking agent DVB. Five encapsulated DNA samples with different degrees of polystyrene linkage were synthesized (Table I). The basic structure of all PS-DVB microcapsules is identical (Figure 1). REM pictures of samples **C1–C5** show the nearly globular structure of the PS-DVB microcapsules with diameters between 1 and 5 μm (Figure 2). Several capsules are associated in grape-like bunches, which may, however, be an artifact of microscopic preparation and drying.

Precision and Accuracy for Inhibition Assays

An inhibition examination was accomplished because qPCR measurements of the ssDNA were performed directly after ssDNA extraction without further DNA purification. Intra- and interassay variation showed no influence of the digestion solution or the TSB-mixture on c_q-values of ssDNA concentrations

Table III. Inhibition Control with TSB-Solution and Sample C6

c_q for employed DNA concentrations							
DNA	100 pg	10 pg	1 pg	10 fg	1 fg	R^2	E
Intra	6.27 ± 0.22	10.02 ± 0.00	13.48 ± 0.01	20.52 ± 0.22	23.79 ± 0.15	0.999	93.1%
Inter	6.10 ± 0.27	9.80 ± 0.25	13.28 ± 0.23	20.33 ± 0.25	23.54 ± 0.30	0.998	93.3%
Absolute	–	9.72 ± 0.23	13.23 ± 0.20	20.34 ± 0.20	23.68 ± 0.32	0.999	92.3%

Abbreviations: intra, intra assay variation, measured with one sample in duplets; inter, inter assay variation, measured with two samples in duplets; absolute, absolute quantification, correlation with standards.
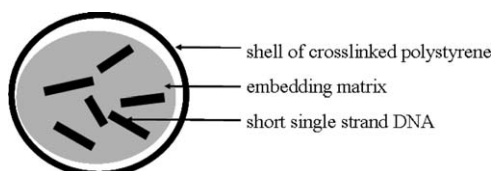
Figure 1. Basic structure of encapsulated DNA (Embedding matrix consists of a 4.7% crosslinked polyacrylamide/water matrix).

in a range between 10 pg and 1 fg for the encapsulated DNA samples (Tables II and III). The measured absolute ssDNA concentration was compared with the employed standards, demonstrating that neither the digestion solution itself nor TSB enhance or inhibit the absolute DNA quantification (Tables II

and III). As a result direct qPCR-measurement from the digestion solution was possible.

DNA Release from PS-DVB Microcapsules

By calculating the ratio of DNA concentration before and after DNA release, we proved whether the DNA is located in the inner core of the PS-DVB microcapsules or not. The DNA concentration was found to be 7.7-fold higher after the destruction of the shell than before (data not shown). Therefore, we concluded that the major part of the DNA is encapsulated and located in the core of the microcapsules. Nevertheless, a part of the ssDNA seemed to be adsorbed at the outside of the shell, because the batches showed low ssDNA amounts before capsule destruction as well. It must be considered, that there is no
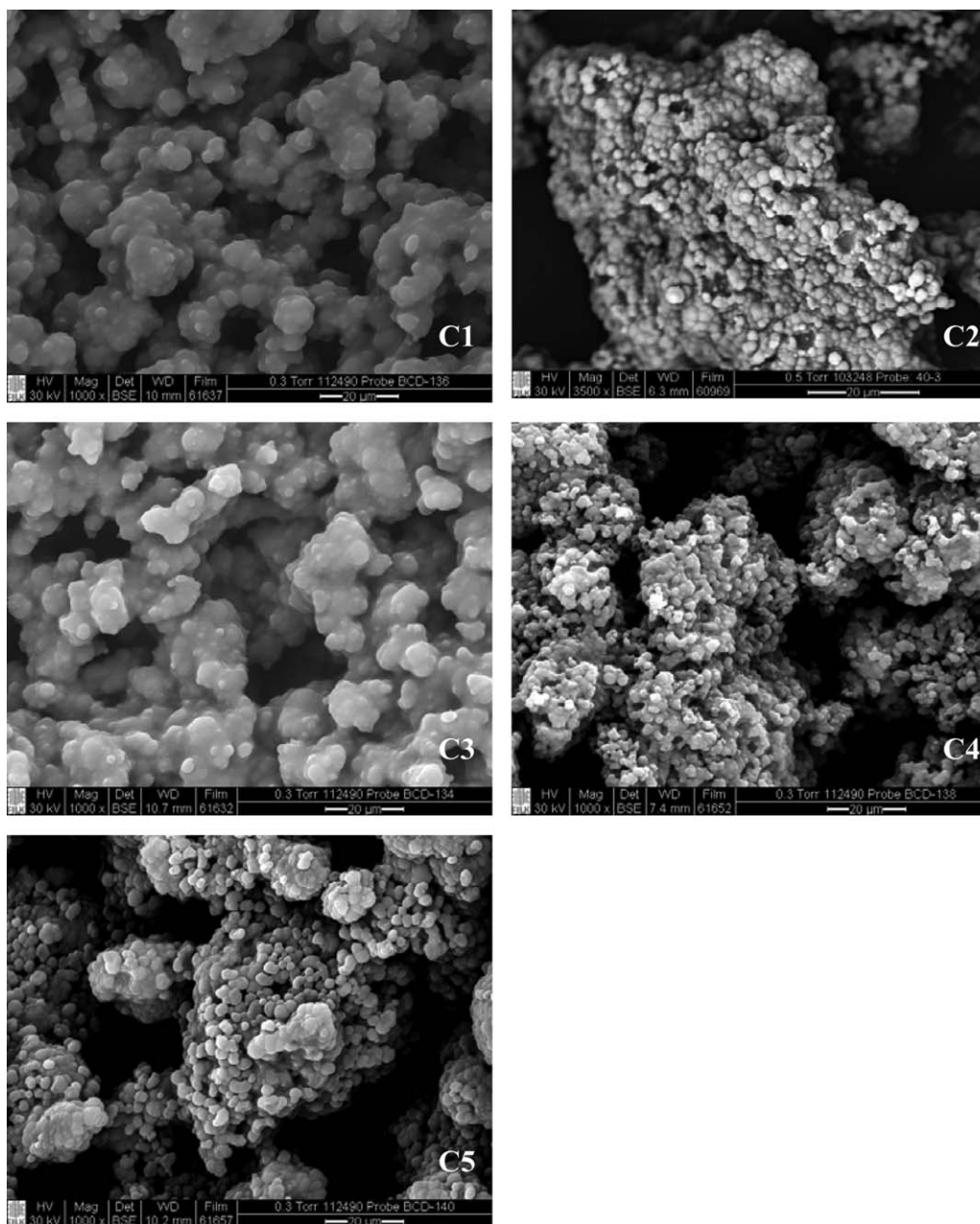

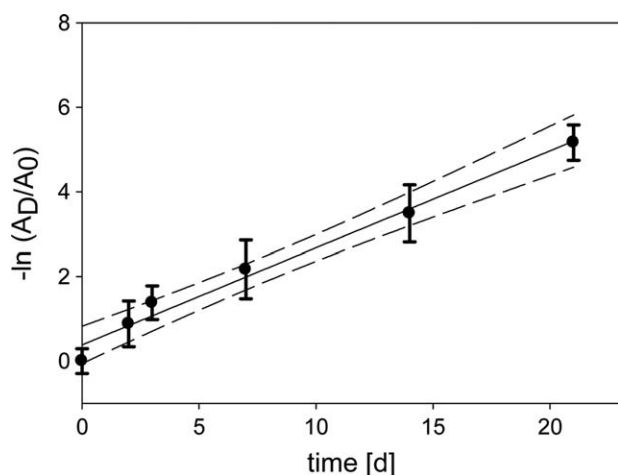**Figure 2.** Scanning electron microscope pictures of samples C1–C5.

Figure 3. Time-dependent amplification ratio (A_D/A_0) for encapsulated ssDNA (C1) in acidic solution (pH 2.0) (without digestion). The solid line is the linear regression; the dashed lines are the 95%-confidence band.

Table IV. Amplification Ratios for C1 as a Function of Digestion and Associated Rate Constants k and Half-Lives $t_{1/2}$

Time (d)	R_A (C1, no digestion)	R_A (C1, digestion)
0	1.00 ± 0.15	1.00 ± 0.27
2	0.41 ± 0.11	0.40 ± 0.12
3	0.25 ± 0.05	0.24 ± 0.07
7	0.11 ± 0.04	0.10 ± 0.03
14	0.03 ± 0.01	0.03 ± 0.01
21	0.01 ± 0.00	0.01 ± 0.00
k (d^{-1})	0.23 ± 0.01	0.22 ± 0.02
$t_{1/2}$ (d)	3.01 ± 0.26	3.15 ± 0.59

guaranty for equal DNA concentration in all capsules. Therefore, all measured DNA amounts were normalized to an untreated sample.

DNA Stability

In literature, DNA decay is described as a natural decay, for example, for double-stranded DNA, which was exposed to UV radiation or for hydrogen peroxide-induced DNA damage.[11,14,15] Allentoft et al. also describe in situ DNA decay by a first-order kinetics.[16] Likewise, in the experiments presented here a fitting with first-order kinetics shows the best regression and therefore provided the basis for the calculation of the apparent half-life and rate constant.

The DNA stability of the encapsulated ssDNA of sample **C1–C5** was checked in acidic TSB (pH 2.0). The ssDNA containing PS-DVB microcapsules of **C1** were incubated at pH 2.0, afterward the ssDNA was extracted. Because abasic sites reduce the amplification efficiency,[17] we included a digestion step in our analytics. The obtained ssDNA was digested with the restriction enzyme endonuclease V, which cleaves DNA at abasic sites. Before and after the digestion step a qPCR was performed, from which R_A was calculated. Figure 3 shows the detected amplification ratio of ssDNA from **C1** at pH 2.0 against time without the digestion step. The rate constant of the ssDNA decay of **C1** at pH = 2.0 is 0.23 ± 0.01/day, the half-life is 3.01 ± 0.26 days (Table IV). With digested DNA from **C1** we obtained the same curve shape and therefore the same rate constant and half-life within the standard deviation (Table IV). Abasic sites spontaneously degrade to strand breaks.[7] The average half-life of an abasic site is 190 h (in vivo, 37°C).[18] Therefore, formed abasic sites spontaneously degrade during the chosen time interval of 21 days, especially at acidic pH, and no digestion assay is required for encapsulated ssDNA.

Non-encapsulated ssDNA has an apparent half-life of 0.40 ± 0.10 days in acidic TSB of pH 2.0[12] (Table V). Hence, only half of the initial DNA amount is still detectable by qPCR after about 9.6 h in TSB of pH 2.0. A successful protection of ssDNA against acidic pH-values should mainly depend on the tightness of the PS-DVB microcapsules. For the microcapsules **C1–C5** the time-dependent amplification ratio shows decay curves at pH = 2.0 (Figures 3 and 4). The high standard deviations are probably due to the normalization over sample size. An internal normalization is not possible with this assay, because an internal standard would also be damaged at pH 2.0. The rate constants, the half-lives for every sample and the associated regression coefficients are specified in Table V. Because the calculated half-life for encapsulated ssDNA is increased compared with non-encapsulated ssDNA ($t_{1/2} = 0.4$ day), we

Table V. Overview of the Rate Constants (k), Half-Lifes ($t_{1/2}$), and Coefficients of Determination (R^2) of Encapsulated ssDNA at pH = 2.0

sample	Proportion of DVB in the polystyrene shell	R^2	k (d^{-1})	$t_{1/2}$ (d)
ssDNA, digested[a]	–	0.93	1.74 ± 0.21	0.40 ± 0.10
ssDNA, not digested[b]	–	0.92	0.58 ± 0.10	1.20 ± 0.42
C1, digested	25%	0.98	0.22 ± 0.02	3.15 ± 0.59
C1, not digested	25%	0.98	0.23 ± 0.01	3.01 ± 0.26
C2	40%	0.98	0.19 ± 0.01	3.65 ± 0.34
C3	50%	0.99	0.12 ± 0.00	5.78 ± 0.45
C4	75%	0.98	0.17 ± 0.01	4.08 ± 0.48
C5	100%	0.97	0.15 ± 0.01	4.62 ± 0.62

[a] Reference with digestion.
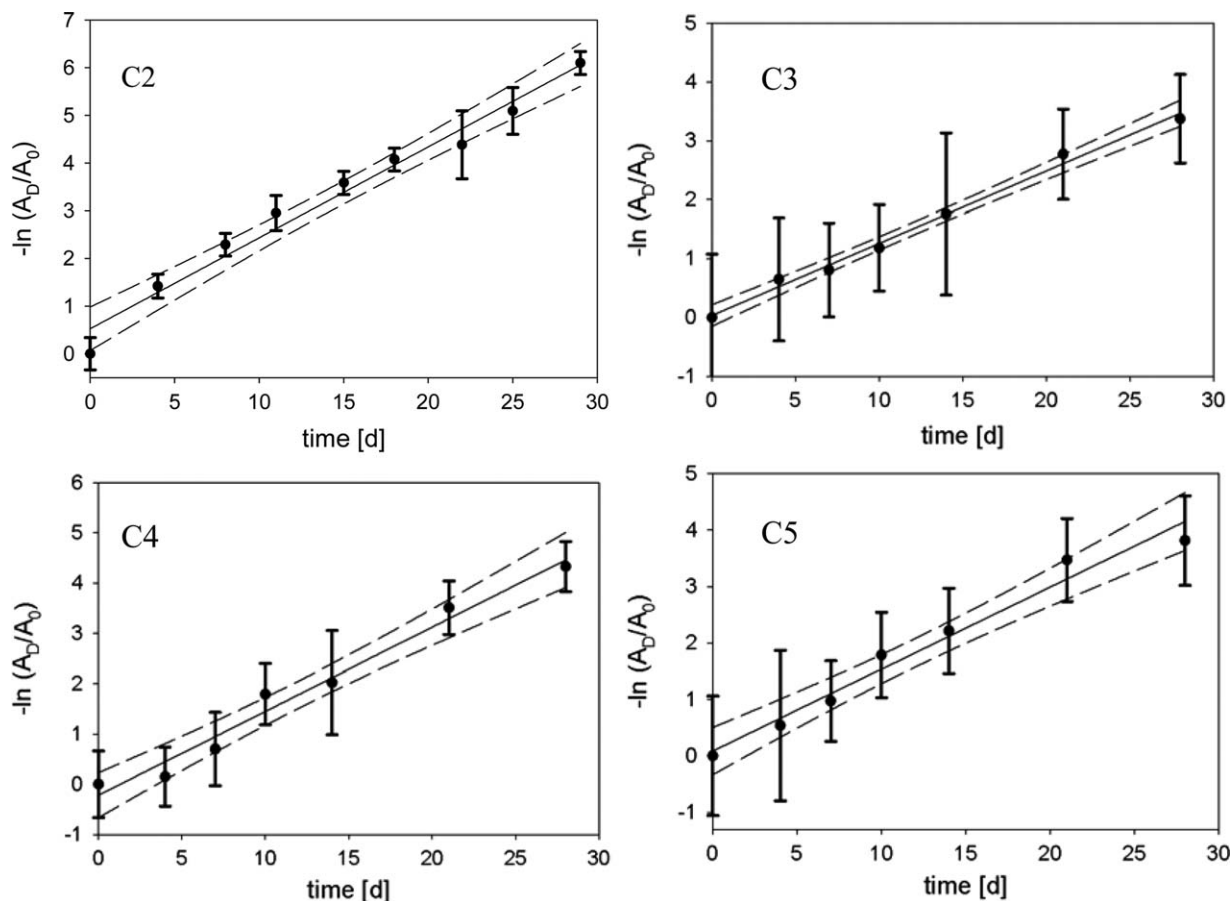[b] Reference without digestion, both from Stenzel and Meyer.[12]

Figure 4. ssDNA decay in polystyrene capsules with different crosslinking degrees. Each point represents the mean value of three samples, measured in duplets, respectively. The *solid line* is the linear regression; the *dashed lines* are the 95%-confidence band.

concluded that the samples **C1–C5** protect the ssDNA in acidic solution against hydrolytic attacks. Therefore, an encapsulation of ssDNA enhances the ssDNA stability at pH = 2.0. The dependence of the rate constant on the content of the crosslinking agent and therefore on the crosslinking degree of the PS-DVB microcapsule is shown in Figure 5. Because a higher rate constant indicates a faster decline of the amplification ratio, the sample with a content of DVB in the shell of 50% (**C3**) shows the best enhancement for

ssDNA stability. A DVB content of 50% leads to a 14-fold increased half-life of the encapsulated ssDNA in contrast to non-encapsulated DNA. Thus, the highest density of the PS-DVB microcapsule is reached by a 50% DVB content in the shell of the microcapsule. A higher DVB content leads to inhibition of the polystyrene crosslinking induced by oversupply of crosslinking agent. Due to the measured DNA damage, it can be concluded that the capsules are not impermeable. The pores of the capsules suggest an open system of exchange, especially of water and therefore also for hydronium ions. A crosslinking degree of the PS-DVB microcapsules of 50% means the smallest pores and therefore the slowest water exchange.
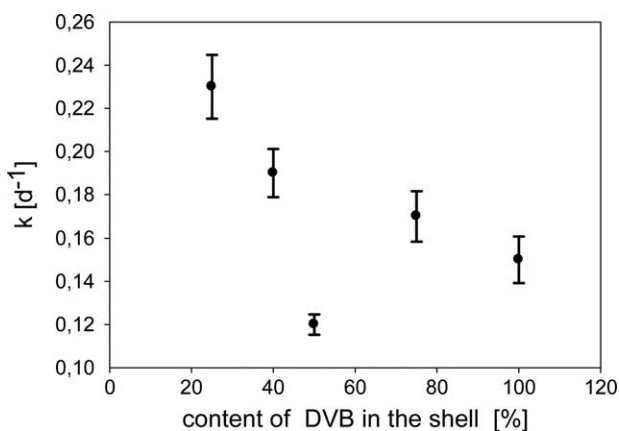
CONCLUSIONS

Common encapsulation applications for drug and gene delivery systems base upon reversible systems, from which the encapsulated ingredient can be easily released. Such reversible systems are, as a small selection, chitosan-based[19] or gelatin-based[20] hydrogels, polyethylenimine, or lipid vesicles[21] (for review see Ref. 22). Thus, the encapsulation of ssDNA with polystyrene is an uncommon concept, because polystyrene is crosslinked irreversibly and therefore a release of ssDNA is only possible by mechanical extraction. But the capsule shell is a physical barrier against hydrolytic attacks, because of its tightness and its hydrophobic character. Therefore, the PS-DVB microcapsule reduces

Figure 5. Dependence of the rate constant on the DVB content in the polystyrene shell.

ssDNA damage induced by acidic influences. The highest ssDNA stability at pH 2.0 is achieved with a PS-DVB shell containing 50% of the crosslinking agent DVB, because the calculated half-life is 14-fold increased in contrast to non-encapsulated ssDNA.

As the protection of ssDNA depends mostly on the pore size of the polystyrene microcapsule, a protection against acidic environments should mean likewise a hindered permeation of larger ions through the capsule pores, such as ions from oxidizing or reducing agents or heavy metal ions and also DNases. Thus a reaction of these ions or enzymes with the inert capsule material polystyrene can be excluded.

The results show that ssDNA stability in acidic pH can be enhanced for short-term application. For this reason polystyrene capsules with the ability to enhance DNA stability could be used not only as tracers in mine waters, but also as marking system for paper, leather, or other biomaterials, where the marking system is exposed to acidic pH only during production. Further investigations concerning heat, humidity, and UV resistance are currently under way.

ACKNOWLEDGMENTS

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