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„DNA Loaded Polymer Nanoparticles for Sub-surface Tracer Technology"

verfasst von / submitted by

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Contents

1. Introduction

1.1 Motivation

There is an increasing demand for robust chemical flow tracer systems to map fluid flow and fluid distributions for various geological reservoirs or to trace wastewater effluent leakage[1] from landfill sites or contaminated surface water. Chemical flow tracers are often used in the oil and gas industry, for instance in hydraulic fracturing treatments[2] (more commonly known as fracking), to map fluid flow or to obtain information about the geology, connectivity and efficiency of drilling sites. The tracer technologies used in the oil & gas industry today can be categorized in two types: (1) Nonreactive, easily differentiated materials that are being injected into the mud circulating system at a certain time to be identified when it returns to the surface. Mud tracers are used to determine circulation times. Dyes, paints, glitter or any material that will follow the mud are typically being used. (2) A chemical or isotopic marker that is uniformly distributed in the continuous phase of a drilling or completion fluid, or installed in a completion string in solid form to dissolve under certain conditions and used to later identify the fluids sampled from the well fluid recovery. The application of any of these methods is limited in that they generally provide a limited number of tracers, i.e. it can be difficult to tag multiple fluids in multiple zones and differentiate them. Moreover, using these methods it is quite difficult to follow long-term events, such as detection of injected water from offset injection wells, because of material degradation. Currently, mathematical modelling[3] is still the primary method of quantifying (estimating) the flow of different fluids within the reservoir or between different reservoir horizons, for example, through fractures or faults, even though the input parameters for the models are incomplete.

Furthermore, environmental protection agencies as well as local communities extracting their water in areas near exploited oil and gas resources, landfill sites or agricultural farms also benefit from tracer technology. For instance, in recent years controversies regarding environmental conflicts between oil companies and local residents have gained much attention. It is often difficult to determine whether a contamination is related to a company's drilling activity or to other causes, such as

agricultural activities, sewage leakage or run-off from farmed land. A famous case was the Range Resources' Carter Impoundment. The Pennsylvania state Department of Environmental Protection (USA) admitted that they used incomplete evidence to determine whether contaminants leaking from a wastewater storage site containing drill cuttings of an exploration site in the Marcellus Shale contaminated a water well and springs in the area*. Clearly, if a suitable flow tracer system could have been used during the drilling and fracking process or would have been added to the wastewater storage site, such accusation would have been easily resolved.

1.2 State-of-the-art

A multitude of different tracer systems is already in use for instance in medicine[4] or biology but also water management and the oil and gas industry. Examples of tracer systems include dye tracers[5], which are harmless to the environment and detectable at very low concentrations but consistently being adsorbed on rocks within the reservoir.[6] Furthermore, various radioactively labelled and chemical tracers[7], such as halogens, thiocyanate anions, ammonium nitrate and alcohols[6] are often used. These tracers can be detected at very low concentrations but have certain disadvantages too; they are often harmful to the operators and/or the environment.

DNA has been already used as a tracer in various applications. It provides numerous advantages; DNA is unique depending on its sequence, can be detected easily and selectively and it is quite stable under standard conditions.

Sergeyev et al.[8] showed that the cationic amphiphile dimethyldiocta-decyl-ammonium chloride (DODAC) can be utilised to stabilise DNA of various molecular masses by forming complexes with its negatively charged backbone. It is also known from literature, that DNA complexes formed between DNA and amphiphiles containing only one alkyl chain do not dissolve in organic solvents or water. However, dialkyl amphiphiles are able to form DNA/surfactant complexes that do

* Hopey "Pennsylvania DEP admits drilling probe error in Range inquiry: Tainted-water hearing targets Range inquiry" Pittsburgh Post-Gazette link in: http://powersource.post-gazette.com/powersource/companies/2014/09/29/DEP-admits-drilling-probe-error/stories/201409280221 accessed on 02.09.2016.

dissolve in low polar organic solvents.[9] All these studies were performed using double stranded (dsDNA). For instance, Rumschoettel et al.[10] used double stranded Salmon DNA (1.3 x 10^6 g/mol, 2 kbp) to investigate the size of DNA complexed with hyperbranched poly(ethyleneimine). They found loosely packed DNA macromolecules to be observed in the size range between 50 nm and 100 nm. Dias et al.[11] found a mean hydrodynamic radius of about 330 nm for Cliphage T2 DNA (1.1 x 10^8 g/mol, 164 kbp).

Grass et al.[12, 13] very recently reported another way of protecting DNA. They investigated a silica based DNA encapsulation strategy to store information encoded in the DNA for various applications, such as prevention against adulteration of essential oils by tagging products for cosmetic, therapeutic and culinary uses. They used dried unprotected dsDNA and showed that the DNA is stable up to temperatures of 70 °C for one week when encapsulated in silica. In order to release the encapsulated DNA from the silica, toxic hydrogen fluoride was required to dissolve the protective silica shell. Sharma et al.[14] protected dsDNA by encapsulation in polylactic acid (PLA) along with paramagnetic iron oxide nanoparticles. The DNA loaded PLA particles were about 400 – 500 nm in diameter. In small-scale experiments they showed that the tracers can be flushed through porous media and that the tracer particles could be recovered by collecting them after pushing them through a column filled with silica sand (12/20 sieve size) and water. After releasing the DNA from the PLA by adding an equal volume of chloroform, they quantified the DNA using real-time Polymerase Chain Reaction (qPCR) and comparing the resulting Ct values to a standard calibration curve. However, for the intended application of flow monitoring in geological formations, such as oilfield applications, the 400 – 500 nm particles are too big; they would filter against porous rocks. Besides the use of encoded or unique DNA tracer systems, several other tagging technologies were reported including polypeptides[15], magnetic inks[16], fluorescent labels[17], photochromic and thermochromic inks[18], radioactive labelled tracers[19] and Raman-active components[20].

1.3 Preliminary Work conducted in our Group†

The aim of this project was to explore options to encapsulate DNA for use as a tracer for a range of oilfield and groundwater applications. The concept is to inject DNA loaded polymer nanoparticles into oil and gas reservoirs and to collect them from production wells (or even *via* reservoir or downhole sampling), using the unique DNA signatures used for each injector to trace the fluid connectivity within the reservoir and pathways to the collection points.

The requirements for such a tracer system are:

- Conditions for DNA encapsulation to avoid denaturation or any molecular damage to DNA
- Diameter d < 100 nm to facilitate transport through reservoirs of milliDarcy (mD) permeability or below
- The ability of the DNA nanoparticles to withstand typical reservoir conditions (temperatures and pressures, say 200°C, 1000 bar)
- To be able to separate/concentrate recovered DNA nanoparticles from the recovered fluid stream
- Ability to recover intact DNA from the tracer polymer nanoparticles post-capture under relatively mild conditions for subsequent sequence analysis

The development of a uniquely identifiable fluid barcode that can provide millions of codes would address a number of the issues that limit tracer uses today.

Challenges

In 2012 McCann et al. demonstrated a new approach for producing DNA loaded polymer nanoparticles for potential subsurface tracer applications.[21] Nature has used DNA to transfer, store and translate information for millions of years. Free, unprotected DNA, however, is a very labile macromolecule and is easily degraded but if contained (encapsulated) and protected respectively it can withstand extremely harsh conditions. For instance, several extremophile bacteria like pink filamentous

† The preliminary work was conducted by Dr. E. Lam, Dr. M. Tang, Dr. Ch. Schmölzer over a period of 3 years as part of short term consulting projects.

Thermocrinis ruber, the thermophilic cyanobacterium *Synechococcus* or the red algae *Cyanidium caladrium* etc. are reported which are able to thrive and survive extremely harsh conditions, such as extreme pressures, high (T > 80°C) and low (T < -15°C) temperatures, low (pH < 3) and high (pH > 9) pH environments, are tolerant to high salt and dissolved metal concentrations and are hardened to radiation.[22]

Challenge: Conditions for DNA encapsulation to avoid denaturation or any molecular damage to DNA

An elegant way to overcome the drawbacks of free DNA is the complexation of DNA using interactions utilising the negatively charged phosphate groups contained in the DNA backbone, which does not influence the intrinsic information contained in the DNA but significantly enhances its stability.

To synthesise DNA loaded nanoparticles DNA strands need to be as small as possible. However, these DNA strands have to be long enough to contain specific information that can be detected accurately and unambiguously to a very high degree of certainty; it was decided to use single stranded DNA (ssDNA). In order to enable multiple variations of encoding information into DNA and to reduce the possibility of misdetection because of close similarity of base pair combinations, a certain length of the sequence is important. Therefore, 100 bases (100b ssDNA) were chosen. At the 3' and 5' end, respectively, 20 bases were reserved to interact with the primers for detection *via* Polymerase Chain Reaction (PCR). Preliminary findings showed that dimethyldioctadecylammonium bromide (DODAB) was a suitable complexation agent for ssDNA to ensure that the final complex is small enough, hydrophobic to allow for the transfer of the DNA complex into typical monomers and protected against damage during encapsulation.

In conclusion, in was demonstrated that it is possible to hydrophobise DNA so that it can be transferred into a monomer phase. The precise formation of the 100b DNA complex still requires clarification, which will enable the optimisation of the complexation to ensure that most, if not all, complexed DNA is transferred into the monomer phase and sufficiently protected. Originally, the complexation was performed in an aqueous solution containing DODAB and ssDNA, but only low quantities of the complex could be recovered and transferred into the monomer

phase. Improvements were obtained when the DNA was directly added into a DODAB/styrene solution capitalising on the hydrophobisation of the DNA during the complexation step and subsequent transfer into the monomer phase.

Challenge: Ability of DNA nanoparticles to withstand typical reservoir conditions

In order to further enhance the protection of DNA against environmental stresses, such as salt, pH and temperature, the DNA was encapsulated into atactic amorphous polystyrene (aPS) nanoparticles (Fig. 1).

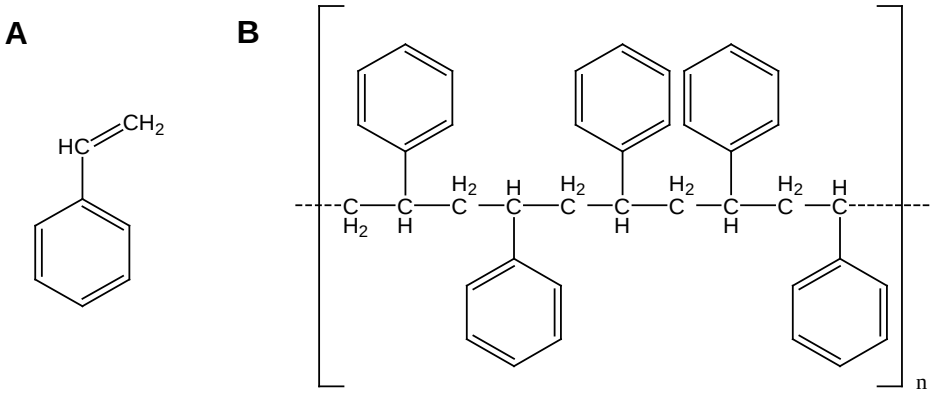


Fig. 1. Structure of **A** styrene and **B** atactic amorphous polystyrene (aPS)

PS has a glass transition temperature (T_g) of approximately 100°C; exposing the particles to temperatures exceeding T_g resulted in deformation and aggregation of the nanoparticles. Moreover, PS is not resistant to organic solvents and oil. Therefore, the PS particles were crosslinked to impart a higher degree of chemical and thermal stability.

The next challenge was to identify a crosslinking agent that allows for the recovery of the ssDNA/DODAB complex from the crosslinked PS nanoparticles for detection. A compound containing a disulphide (S-S) bond, namely bis(2-methacryloyl)oxyethyl disulphide (DSDMA) was chosen (Fig. 2).

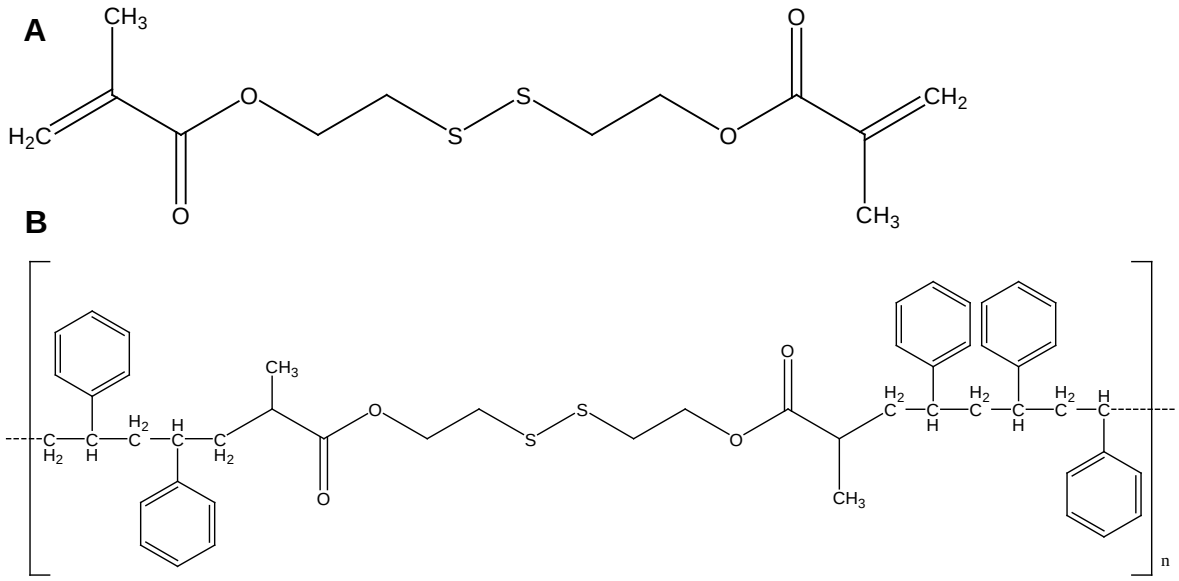


Fig. 2. Structure of **A** the crosslinking agent bis(2-methacryloyl)oxyethyl disulphide (DSDMA) and **B** crosslinked atactic amorphous polystyrene (aPS)

Disulphide bonds provide a good compromise of stability and the possibility of selective cleavage thus enabling subsequent recovery of encapsulated ssDNA (Fig. 3).

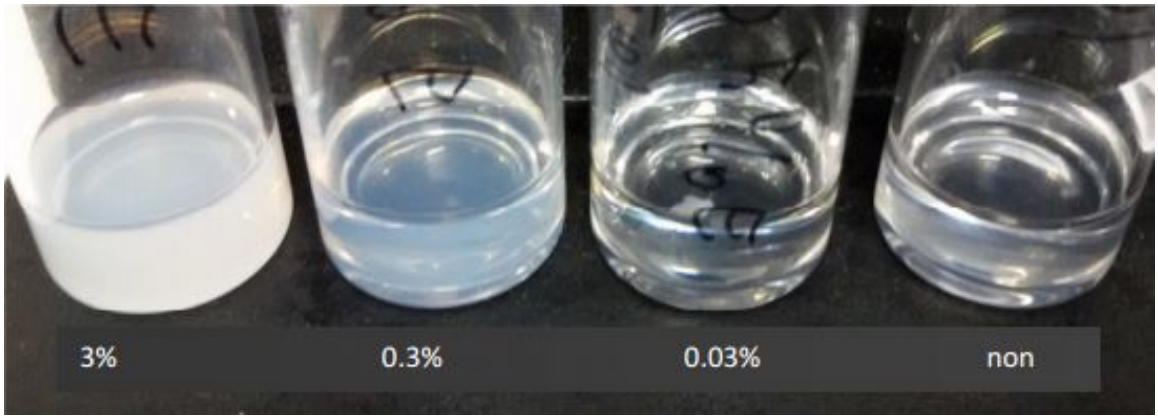


Fig. 3. PS nanoparticles suspended in toluene. On the left hand side the particles contain 3 wt.% of bis(2-methacryloyl)oxyethyl disulphide (crosslinker). From left to right the degree of crosslinking density decreases by a factor of 10, exemplifying the enhanced stability of crosslinked PS nanoparticles to dissolution in organic solvents and oil.

In conclusion, it was shown that DNA could be encapsulated into crosslinked PS nanoparticles, which are resistant to organic solvents and temperatures up to 100°C. However, the survivability of encapsulated DNA at subsurface conditions has yet to be quantified.

Challenge: Diameter d < 100 nm to facilitate transport through reservoirs

DNA loaded PS nanoparticles were synthesised by miniemulsion polymerisation. Prior to forming the miniemulsion the ssDNA was complexed with DODAB in styrene. After complexation the styrene phase was added to the aqueous phase containing the water-soluble initiator 2,2'-azobis(2-methylpropionamidine) dihydrochloride and the mixture was emulsified by ultrasonication. The miniemulsion was stabilised by the surfactant polyethylene glycol 1100 mono(hexadecyl/octadecyl) ether dissolved in the aqueous phase. The polymerisation of the particles was performed by stirring the emulsion at 70°C. To determine the particle size SEM images were captured (Fig. 4).

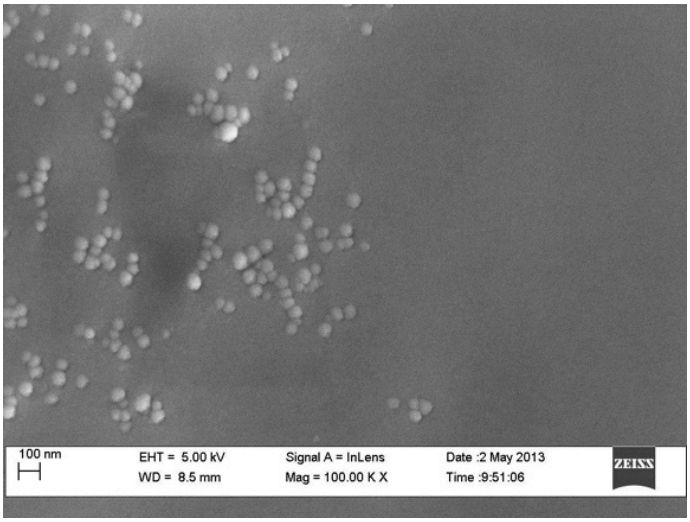


Fig. 4. SEM image of synthesised crosslinked PS nanoparticles after removal of the largest particle fraction by centrifugation; the bar corresponds to 100nm.

In conclusion, it was demonstrated that it is possible to synthesise crosslinked PS nanoparticles with dimensions below 100 nm, which should be suitable for subsurface tracer applications.

Challenge: Recovered intact DNA from the tracer polymer nanoparticles under mild conditions allows for subsequent sequence analysis

In order to recover the DNA from the crosslinked particles, the disulphide bonds of the crosslinker had to be broken under conditions at which DNA survives. In molecular biology various water-soluble reducing agents, such as dithiothreitol (DTT) or tris(2-carboxyethyl) phosphine (TCEP) are used to cleave disulphide bonds; however they were found to be ineffective to reduce the hydrophobic disulphide crosslinked PS-nanoparticles. One of the most common methodologies used to reduce disulphide bonds is the metal mediated reduction with hydrogen. The catalyst of choice was Raney Nickel (RaNi) which was activated by leaching Aluminium out of Nickel/Aluminium alloy with sodium hydroxide. It has been demonstrated that this reaction is compatible with DNA molecules.[23] It was shown that dsDNA is stable in the reducing conditions generated by Raney Nickel.‡

Once the DNA/DODAB complex was released into ethanol, which was used as the medium in which the tracer particles were broken with RaNi and dried in vacuum, it could be easily decomplexed by adding sodium dodecyl sulphate (SDS), which has a higher affinity to the cationic surfactant (DODAB) than the negatively charged DNA. In preliminary studies, the stability of the DNA during the complexation/decomplexation steps *via* gel electrophoresis, using the iBase™ Power System, was proven, indicating no change in the DNA length (Fig. 5).

‡ VICB Communications, *"Identifying the Elusive Protein_DNA Cross-Link"* http://www.vanderbilt.edu/vicb/DiscoveriesArchives/identifying_protein_DNA_crosslink.html accessed 02.09.2016

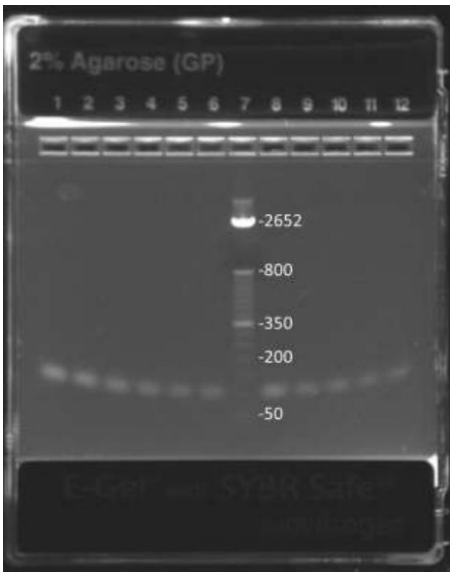


Fig. 5. UV-image taken after running the gel electrophoresis (0.08 s UV exposure); the high salinity of the DNA solutions affects the electrophoretic separation behaviour causing fluctuations in the running behaviour leading to an irregular front.

In conclusion, it was already demonstrated that it is possible to hydrophobise DNA so that it can be transferred into a monomer phase. It was also confirmed that it is possible to break the crosslinked DNA loaded nanoparticles and recover the encapsulated DNA, which can be quantified using gel electrophoresis.

1.4 Objectives

The project aims to provide the fundamental background to effectively synthesise a viable DNA tracer system suitable for subterranean applications with unambiguous detection characteristics. For this purpose, synthetic appropriate single stranded DNA will be encapsulated in a suitable in situ polymerised polymer nanoparticle. Therefor the DNA needs to be complexed with a decent surfactant to be protected against elevated temperatures and to allow for efficient transfer into the monomer phase. Furthermore, miniemulsions are to be formulated and polymerised to produce a stable nanoparticle suspension containing only polymer nanoparticles with dimensions consistently smaller than 100 nm. The Morphology of synthesised DNA loaded particles should be investigated using different methods, such as SEM, AFM, DLS and Nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analysis. DNA encapsulation efficiency should be determined by comparing qPCR results to a standard calibration curve.

2. Experimental Section

2.1 Material

All solvents and chemicals were used as received without further purification (Tab.1).

Reagent	CAS-Number	Purity	Origin	Product-Number
100 base single stranded DNA (lyophilised) Sequence: 5'-GCG CTC TGA GAT CAA GGT GAG GCC TAT TTC TCC AAC AGG AAG CAG AAT TCT AAG CTC TAT ATC TTA AGA GAC TAG GCT CAA CAG GGA AAG GCT AGG GAA A-3'	-	HPLC purified	Integrated DNA Technologies	
2,2'-Azobis(2-methylpropionamidine) dihydrochloride	2997-92-4	97 %	Sigma Aldrich	440914-25G
20 base forward primer (lyophilised) Sequence: 5'-TTT CCC TAG CCT TTC CCT GT-3'	-	HPLC purified	Integrated DNA Technologies	
20 base reverse primer (lyophilised) Sequence: 5'-GCG CTC TGA GAT CAA GGT GA-3'	-	HPLC purified	Integrated DNA Technologies	
Bis(2-methacryloyl)-oxyethyl disulphide	36837-97-5	≥ 98 %	Sigma Aldrich	735094-5G
Cremophor A-25	68439-49-6	-	kindly supplied by BASF	50001118
Dimethyldioctadecyl-ammonium bromide	3700-67-2	96 %	Sigma Aldrich	D2779-10G
Ethanol	64-17-5	96 %	Brenntag	442278
Fast SYBR Green Master Mix	-	-	ThermoFisher Scientific	4385617
Hexadecane	544-76-3	99 %	Sigma Aldrich	H6703-100ML
NH_4OAc	631-61-8	≥ 98.0	Sigma Aldrich	A1542-250G
NiAl alloy	12003-78-0	50 %/50 %	Merck	806749
Sodium dodecyl sulphate	151-21-3	≥ 99.0 %	Sigma Aldrich	L6026-50G
Sodium hydroxide	1310-73-2	99.62 %	ACM	9003680015921
Styrene	100-42-5	≥ 99 %	Sigma Aldrich	S4972-2.5L

Toluene	108-88-3	technical grade	VWR	28701.364
Tris-EDTA buffer solution, pH 7.4	-	-	Sigma Aldrich	93302-100ML
Tween 20	9005-64-5	-	Sigma Aldrich	P2287-100ML
Water (DNase, RNase free)	7732-18-5	-	Sigma Aldrich	W4502-1L

Tab. 1. List of applied chemicals

2.2 Preparation and Characterisation

All shaking processes were done using an IKA MS1 Minishaker (1,200 rpm) and an IKA RCT basic (1,500 rpm) equipped with an IKA ETS-D5 contact thermometer was used for all stirring and heating processes. Emulsification was performed with Hielscher Ultrasound Technology UP100H Ultrasonic Processor (1 cycle, 100 % amplitude, which relates to 100 W and 30 kHz for 20 and 15 minutes respectively). Samples were centrifuged with Thermo SCIENTIFIC ESPRESSO Centrifuge (14,500 rpm for 30 and 5 minutes respectively for washing the particles and 5,000 rpm for 5 minutes for the particle breaking process) and freeze-dried using Telstar LyoQuest.

The particle size and droplet size of the emulsion, respectively, were determined by Dynamic Light Scattering (DLS, Sympatech, Nanophox). Therefor the particle suspension (as prepared in process 3.5, 2 µL) was diluted with distilled water (2 mL) to obtain particle concentration of 2 x 10^{11} particles/mL.

In order to count and size individualised particles, Nano Electrospray Gas-phase Electrophoretic Mobility Analysis (nES GEMMA, TSI Inc. Shoreview, MN, USA) was performed. The particle suspension was used as prepared (process 3.5) and diluted using an aqueous solution of ammonium acetate (50 mM, pH 8.4 including 0.001 (v/v) % Tween 20) to obtain particle concentration of 4 x 10^{12} particles/mL in electrolyte. The analysis was done using

The particle morphology was characterised by scanning electron microscopy (SEM, Zeiss Supra 55 VP, Oxford Instruments X-Max 80) of freeze-dried particles and atomic force microscopy (AFM, Bruker, Nanoscope VIII) of particles separated and collected onto an AFM carrier using nES GEMMA.

To qualify and quantify DNA real-time polymerase chain reaction (qPCR, StepOnePlus™ Real-Time PCR System) was performed and analysed by comparing the resulting Ct values to a standard calibration curve.

PCR plates (4346907), PCR plate sealing films (4306311) used for qPCR are purchased from Applied Biosystems™ by Thermofisher Scientific.

The experiments throughout this thesis were performed 70 times with varying parameters to improve DNA and particle behaviour step-by-step. In the following, only the best working method and results are discussed.

2.3 Preparing DNA and Primer Stock Solutions

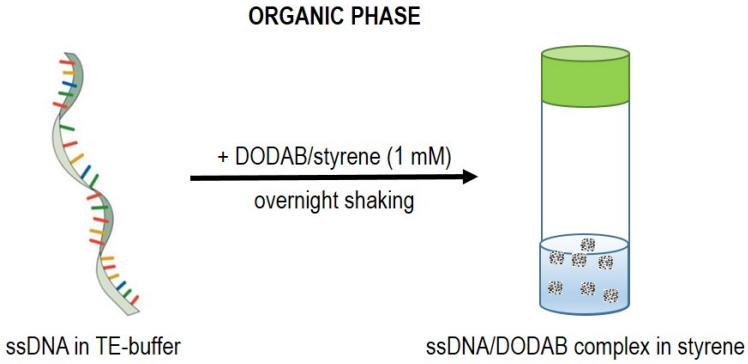
Reagents:

Reagent	Molecular Weight [g/mol]	Mass	Volume	Amount of Substance
100b ssDNA (lyophilised)	31,005.1	0.74 mg	-	23.8 nmol
20b Forward primer (lyophilised)	5,960.9	0.19 mg	-	31.8 nmol
20b Reverse primer (lyophilised)	6,182.1	0.19 mg	-	30.5 nmol
TE-buffer	-	-	1,584 µL	-

23.8 nmol 100b ssDNA were dissolved by adding 238 µL TE-buffer into the DNA containing vial. The resulting solution (100 µM) was separated in equal parts of ~ 50 µL and stored in 200 µL Eppendorf vials (PE) at - 25°C.

31.8 nmol 20b forward primer and 30.5 nmol 20b reverse primer were dissolved by adding 736 µL and 610 µL TE-buffer respectively into the primer containing vials, the resulting solutions (50 µM) were separated in equal parts of ~ 50 µL and stored in 200 µL Eppendorf vials (PE) at - 25°C.

2.4 Complexation of ssDNA

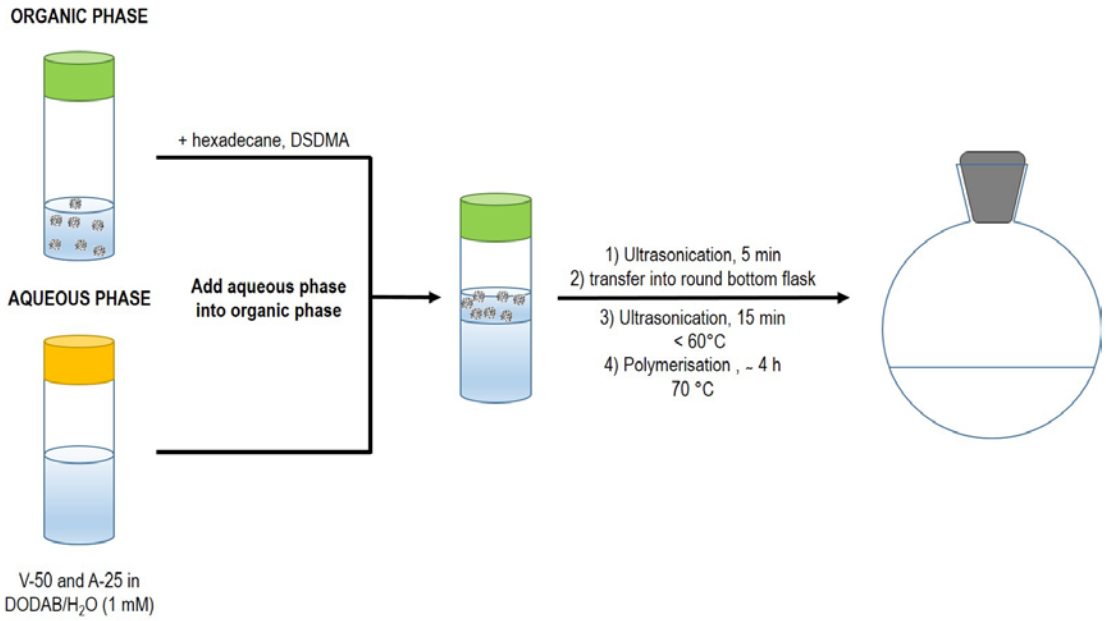


Reagents:

Reagent	Molecular Weight [g/mol]	Volume
100 b ssDNA/TE-buffer (100 µM)	31,000.0	3 µL
DODAB/styrene (1 mM)	630.95	800 µL

In a 15 mL centrifuge tube (PE with PP cap) 100b ssDNA/TE-buffer (100 µM, 3 µL) were added into a solution of DODAB in styrene (1 mM, 800 µL). The mixture was shaken at 1200 rpm for 18 hrs.

2.5 Encapsulation of ssDNA/DODAB Complex and Formation of crosslinked Polystyrene (cPS) Nanoparticles



Reagents:

Reagent	Molecular Weight [g/mol]	Mass	Volume
Hexadecane	226.44	-	34 µL
DSDMA	290.40	-	50.8 µL
V-50	271.19	13.0 mg	-
Cremophor A-25	270.49	171.0 mg	-
DODAB/water (dist.) (1 mM)	-	-	3 mL

Hexadecane (34 µL) and DSDMA (50.8 µL) were added into the 15 mL centrifuge tube containing the organic phase as prepared in process 3.4 and shaken for another 5 min. The aqueous phase was prepared by dissolving the biodegradeable cosmetic grade oil-in-water emulsifier A-25 (171.0 mg) in a solution of DODAB in distilled water (1 mM, 3 mL) and addition of the thermally activated azostarter V-50

(13.0 mg). After adding the organic phase into the aqueous phase, the mixture was sonicated (1 cycle, 100 % amplitude, 20 min). Afterwards the emulsion was transferred into a round bottom flask and sonicated for another 15 min (1 cycle, 100 % amplitude) while cooling using an ice bath to keep the temperature below 60°C. The round bottom flask was equipped with an ellipsoidal magnetic stirring bar and the emulsion was polymerised at 70°C (oil bath) while stirring vigorously (1,500 rpm). 4 hours later the suspension was cooled to room temperature under streaming tap water and freeze-dried if required.

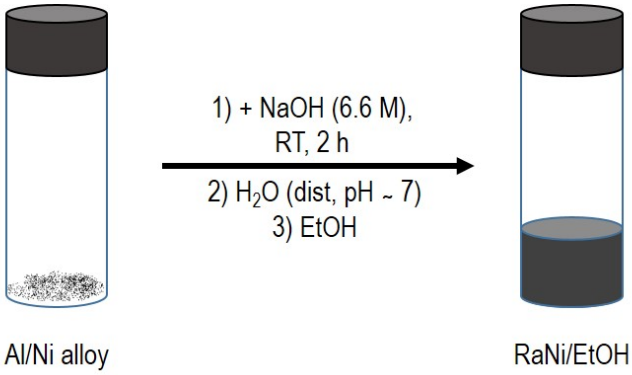
2.6 Washing the Particles

Reagents:

Reagent	Molecular Weight [g/mol]	Mass	Volume
Particles (freeze-dried)	-	~ 750 mg	-
SDS/water (dist.) solution (1 %)	-	-	10 mL
EtOH	46.07	-	10 mL

Freeze-dried particles were resuspended in aqueous SDS solution (1 %, 10 mL) and stirred vigorously. After two hours, the suspension was centrifuged (14500 rpm, 30 min) and the supernatant was removed. Particles were resuspended in EtOH (10 mL), stirred for another two hours and centrifuged (14500 rpm, 5 min). Collected supernatants and particles were dried in vacuum.

2.7 Preparation of Raney Nickel (RaNi)



Reagents:

Reagent	Molecular Weight [g/mol]	Mass	Volume
NiAl alloy	-	60 mg	-
NaOH (aq., 6.6 M)	40.00	-	0.3 mL
Water (dist.)	18.02	-	70 mL
EtOH	46.07	-	3 mL

NiAl alloy (60 mg) was weighed into a 6 mL glass vial with PE cap and aqueous NaOH solution (6.6 M, 0.3 mL) was added. The vial was kept open at room temperature, so the evolving hydrogen gas could pass off. After 2 hours distilled water (0.5 mL) were added and the solid was allowed to settle down. The supernatant was decanted and RaNi was washed with distilled water to neutral pH (~ 10 times with 0.7 mL each). EtOH (3 mL) was added and the suspension was kept tightly closed at room temperature overnight.

2.8 Releasing ssDNA/DODAB Complex

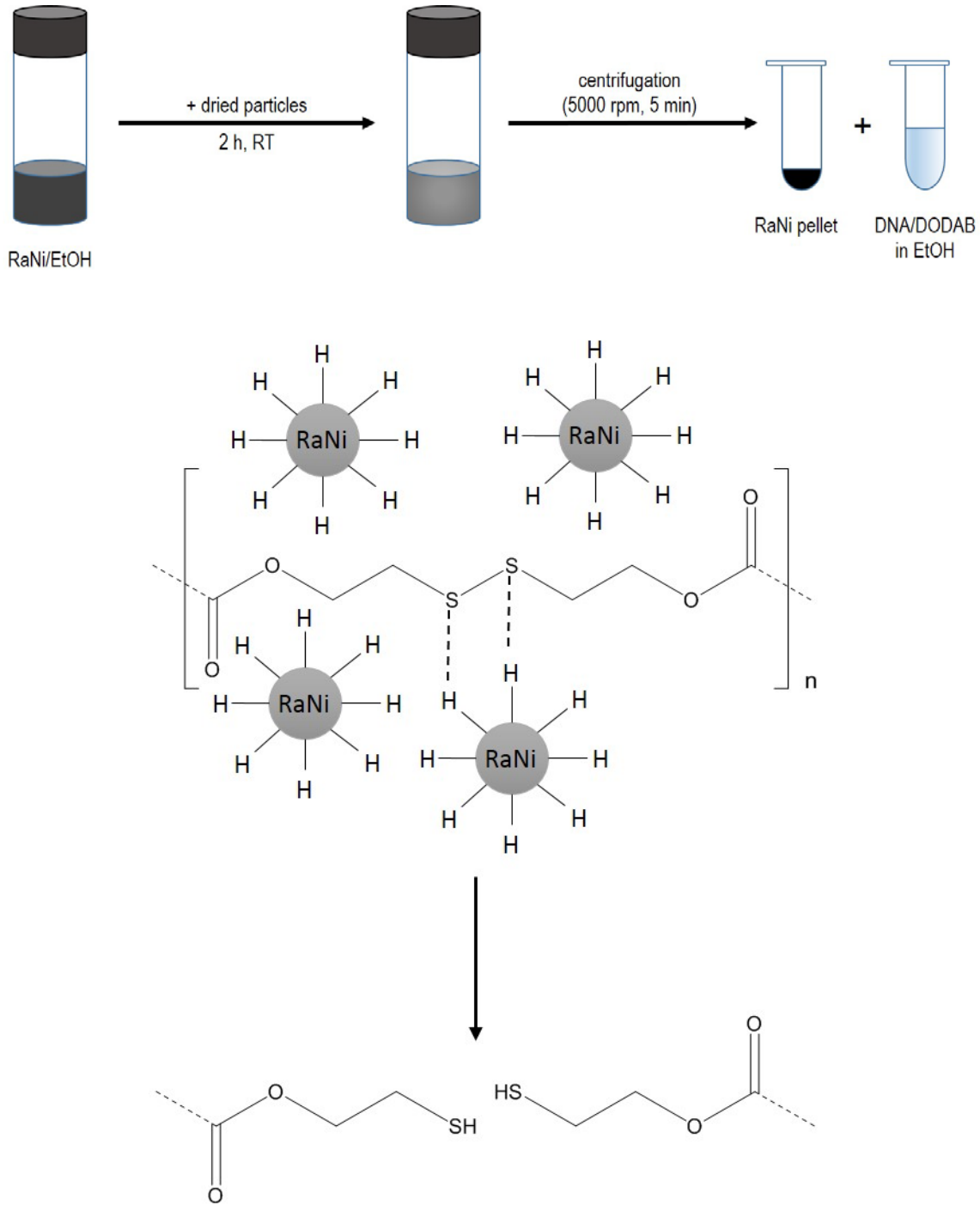


Fig. 5. Schematic mechanism of RaNi mediated cleavage of the disulphide bond present in the crosslinker

Reagents:

Reagent	Molecular Weight [g/mol]	Mass	Volume
Particles (freeze-dried)	-	40 mg	-
RaNi/EtOH	58.69	-	3 mL
EtOH	46.07	-	3 mL

40 mg of freeze-dried particles were weighed into the vial containing the RaNi/EtOH suspension and equipped with a magnetic stirring bar. The mixture was stirred vigorously for 2 hours to cleave the disulphide bonds of the crosslinker (Fig. 6). The suspension was transferred into a centrifuge tube. After centrifugation (5,000 rpm, 5 min) the supernatant was decanted and the remaining RaNi pellet was resuspended in EtOH (3 mL). The suspension was vortexed intensively for 30 seconds and shaken for 2 hours. After another centrifugation (5,000 rpm, 5 min) the supernatant was combined with the first one and the solvent was removed under reduced pressure.

2.9 Qualification and Quantification of ssDNA

In order to determine the amount of ssDNA decomplexing the ssDNA/DODAB complex is necessary. This was done by adding aqueous SDS solution (0.01 %, 500 µL) to each sample including the dried washing solutions resulting from process 3.6 and the dried complex released from particles from process 3.9.

Forward and reverse primer were diluted to 10 µM and 1 µM respectively and all components needed for qPCR were combined into a 196-well plate (Tab. 2).

Component	Volume [µL]	Final Concentration
2X Fast SYBR® Green Master Mix	10	1X
Forward Primer (10 µM)	0.6	6.0 µM
Reverse Primer (1 µM)	0.6	0.6 µM
Template DNA	2.0	≤ 500 ng/reaction
RNase/DNase free water	6.8	
Total reaction volume	20	

Tab. 2. Volumes and concentrations of components to qualify and quantify DNA using qPCR.

The plate was sealed with an adhesive sealing film and analysed using the following program (Tab. 3).

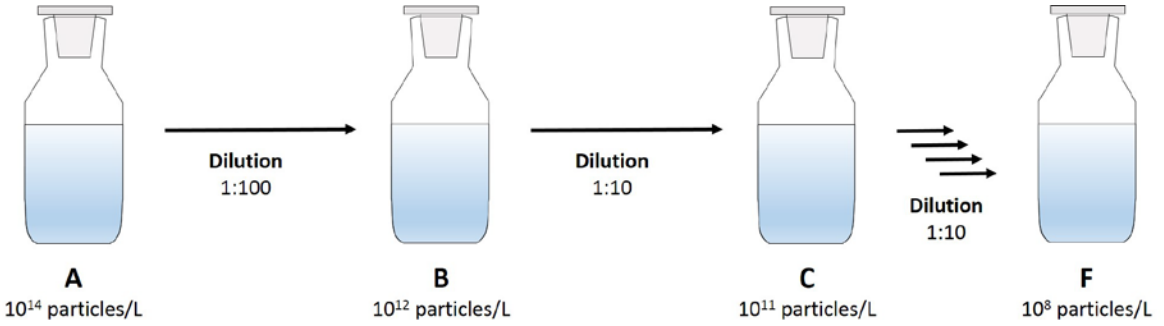
Step	Temperature [°C]	Duration [sec]	Cycles
AmpliTaq® Fast DNA Polymerase, UP Activation	95	20	HOLD
Denature	95	3	40
Anneal/Extend	52	30	

Tab. 3. Fast SYBR® Green Master Mix Protocol. Temperatures and durations of each step of qPCR measurement.

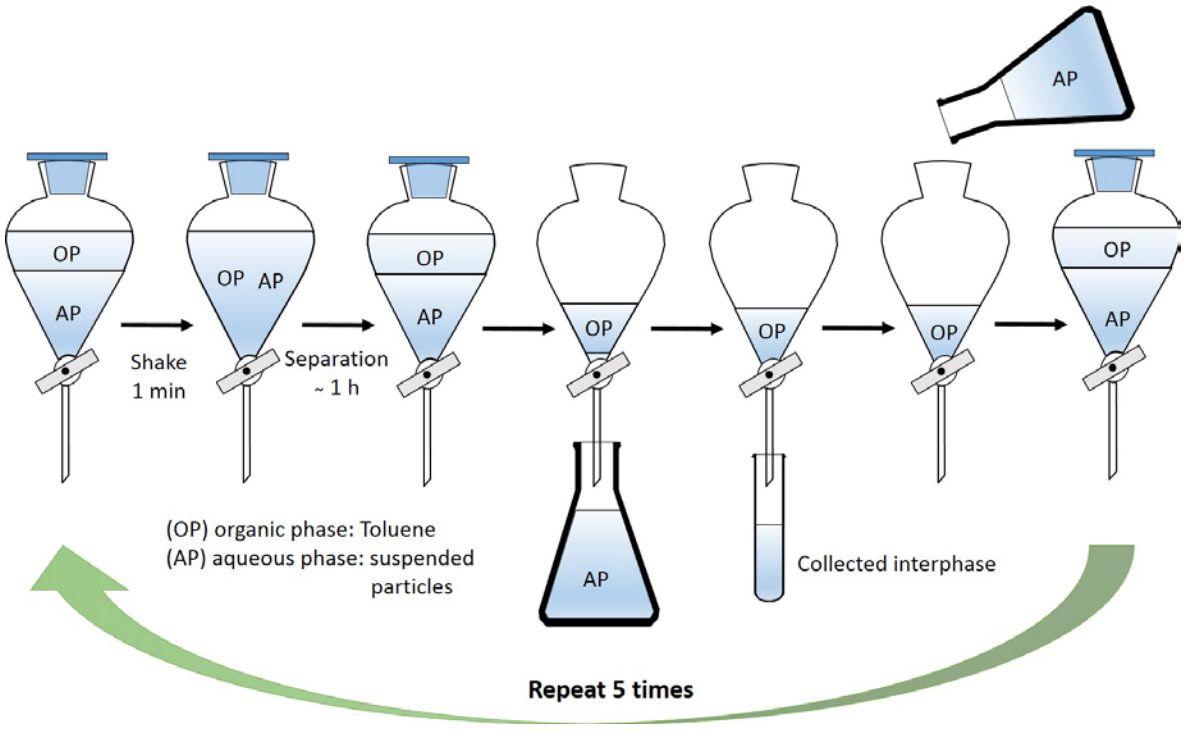
2.10 Separation and Concentration of DNA loaded Particles

Reagents:

Reagent	Molecular Weight [g/mol]	Volume
Particle suspension (as prepared in process 2.5)	-	0.4 mL
Water (dist.)	18.02	7,389.6 mL
Toluene	92.14	2,000 mL



In a 1.5 L bottle (PE with PP cap), 0.4 mL of particle suspension were diluted with water (dist., 999.6 mL) to obtain a concentration of 10^{14} particles/L ("sample A"). 100 mL of this dilution were transferred into another bottle (PE with PP cap) and diluted using water (dist., 900 mL) to get "sample B". The following dilutions were prepared by diluting 10 mL of "sample B" with water (dist., 990 mL) to get "sample C", then diluting 10 mL of "sample C" with water (dist., 990 mL) to get "sample D". This process was continued to maintain a dilution series down to 10^{8} particles/L ("sample F").



Dilution "sample A" (500 mL) was transferred into a glass separation funnel (2 L), combined with toluene (250 mL) and shaken manually for 1 min. After one hour, the

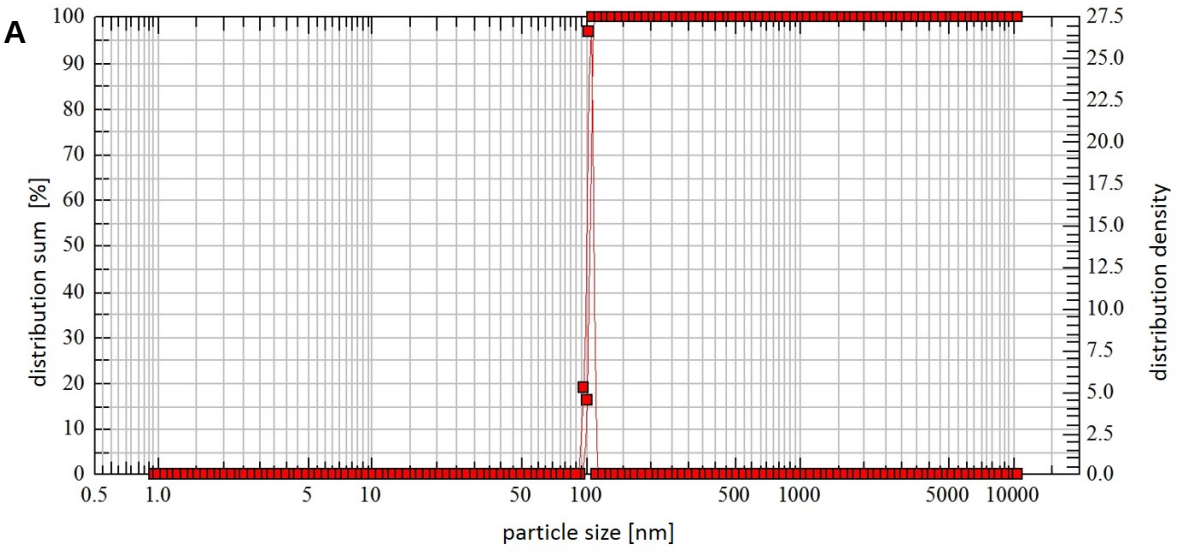
phases had been separated. The aqueous phase was drained into an Erlenmeyer flask and the interphase (emulsion) into a 50 mL Falcon tube (PE with PP cap). Aqueous phase was re-added to the toluene and the process was repeated for another four times. Afterwards the whole extraction procedure was done with “sample B” to “sample F”. The collected interphases were dried in vacuum and the particles were broken by adding RaNi suspended in EtOH following process 2.8.

3. Results and Discussion

3.1 Characterisation of Miniemulsion Templates

Dynamic Light Scattering (DLS)

Prior to the polymerisation process, the monomer droplet size of the emulsion was determined using DLS (Fig. 7). Droplet sizes of ~ 100 nm were found right after emulsification *via* ultrasonication and were stable up to a minimum of 5 min after emulsification. As shown below, polymerized particles are ~ 53 nm in diameter (nES GEMMA, point 3.2). This finding indicates that the droplets of the emulsion are not fusing but flocculating. Due to the vigorous stirring of the emulsion during the polymerisation process, the droplets were separated again.



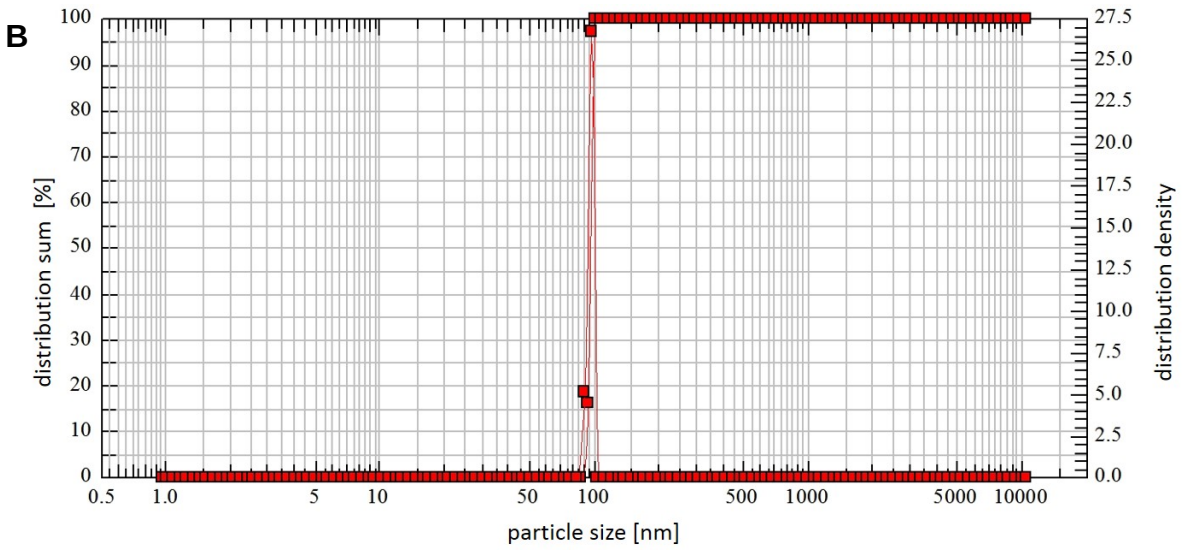


Fig. 7. Typical particles size distribution of droplets of the miniemulsion **A** right after emulsification and **B** 5 minutes later without any further treatment

3.2 Particle Morphology

Scanning Electron Microscopy (SEM)

To determine the particle size, SEM images were taken. Due to the freeze-drying process, the particles agglomerated. Nevertheless, it was possible to determine the dimensions of a number of particles, which were at the surface of the agglomerate (Fig. 8). The particles had diameters ranging from 72 nm up to 103 nm.

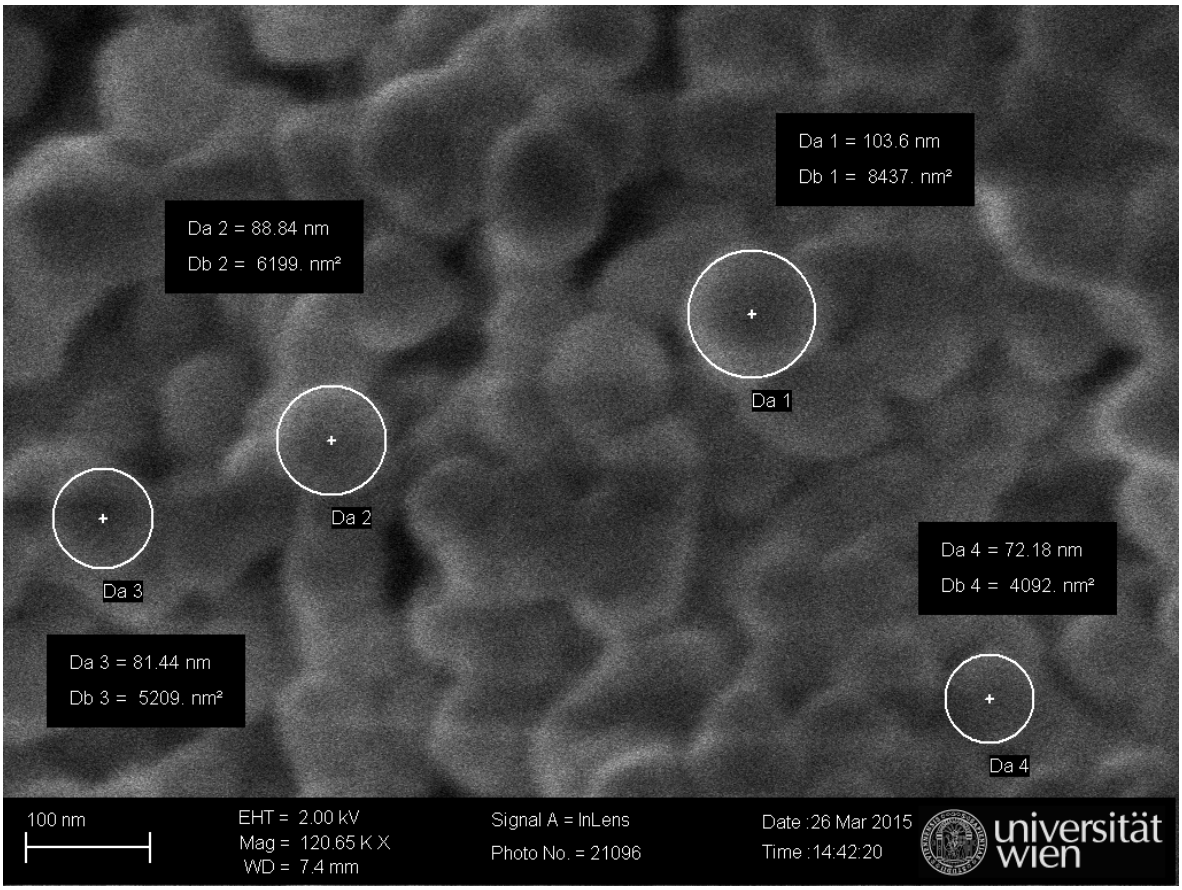


Fig. 8. SEM image showing particles agglomerated due to the freeze-drying process. The particles had diameters ranging from 72 nm up to 103 nm.

Particle Sizing by Nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analysis

In order to size individualised particles Nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analysis (nES GEMMA) analysis was performed. Particles of varying particle diameters were identified (Fig. 9). The majority of particles were 52.5 ± 0.5 nm in diameter. The graph also shows that smaller particles of 22.9 ± 1.0 nm were present. These smaller particles are most likely DNA-DODAB complexes, which remained in the as synthesised particle suspension.

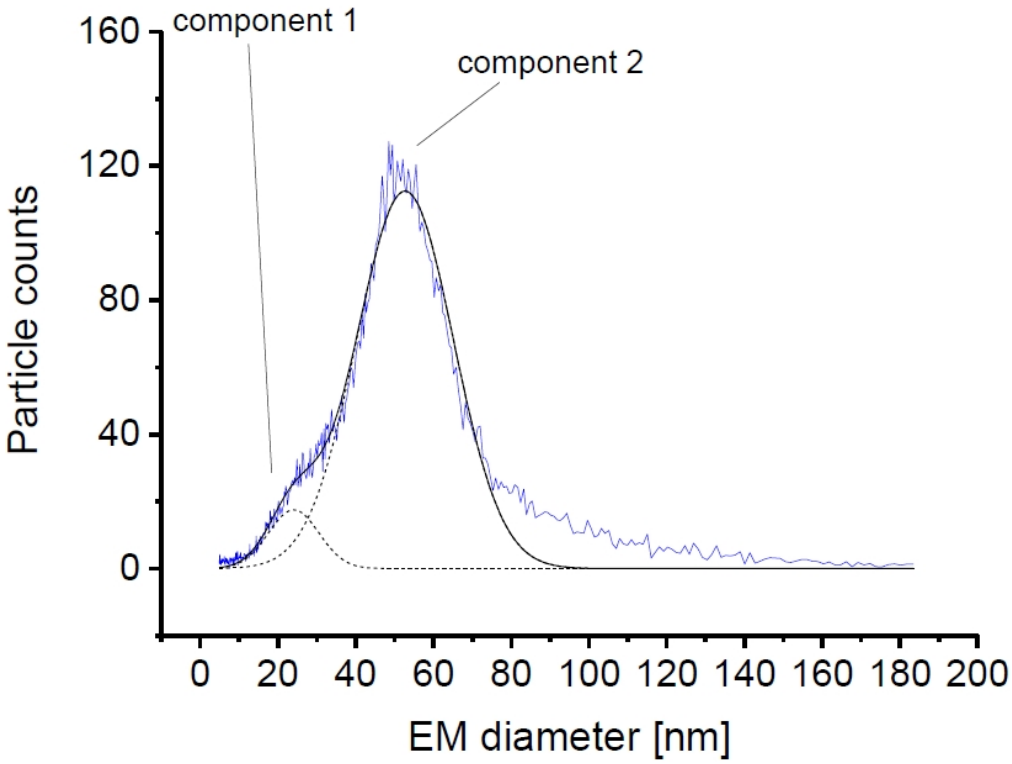


Fig. 9. Particle number concentration based on data/detector channel showing a majority of particles of 52.5 ± 0.6 nm (component 2) in diameter and also smaller particles of 22.9 ± 1.0 nm (component 1) including demonstration of a Gauss peak fitting to nES GEMMA spectra.

Particle sizing by Atomic Force Microscopy (AFM)

The particles classified and sized by GEMMA were collected directly onto an AFM substrate. Those particles were characterised by AFM. The particles had diameters of approximately 100 nm. Taking into account that ~ 53 nm diameter were found using nES GEMMA and looking the elevations profile, it appears that two particles agglomerated (Fig. 10). Furthermore, the elevation profile shows only a height of ~ 5.4 nm, which is due to the fact that the particles are embedded into a layer of dried ammonium acetate and Tween 20.

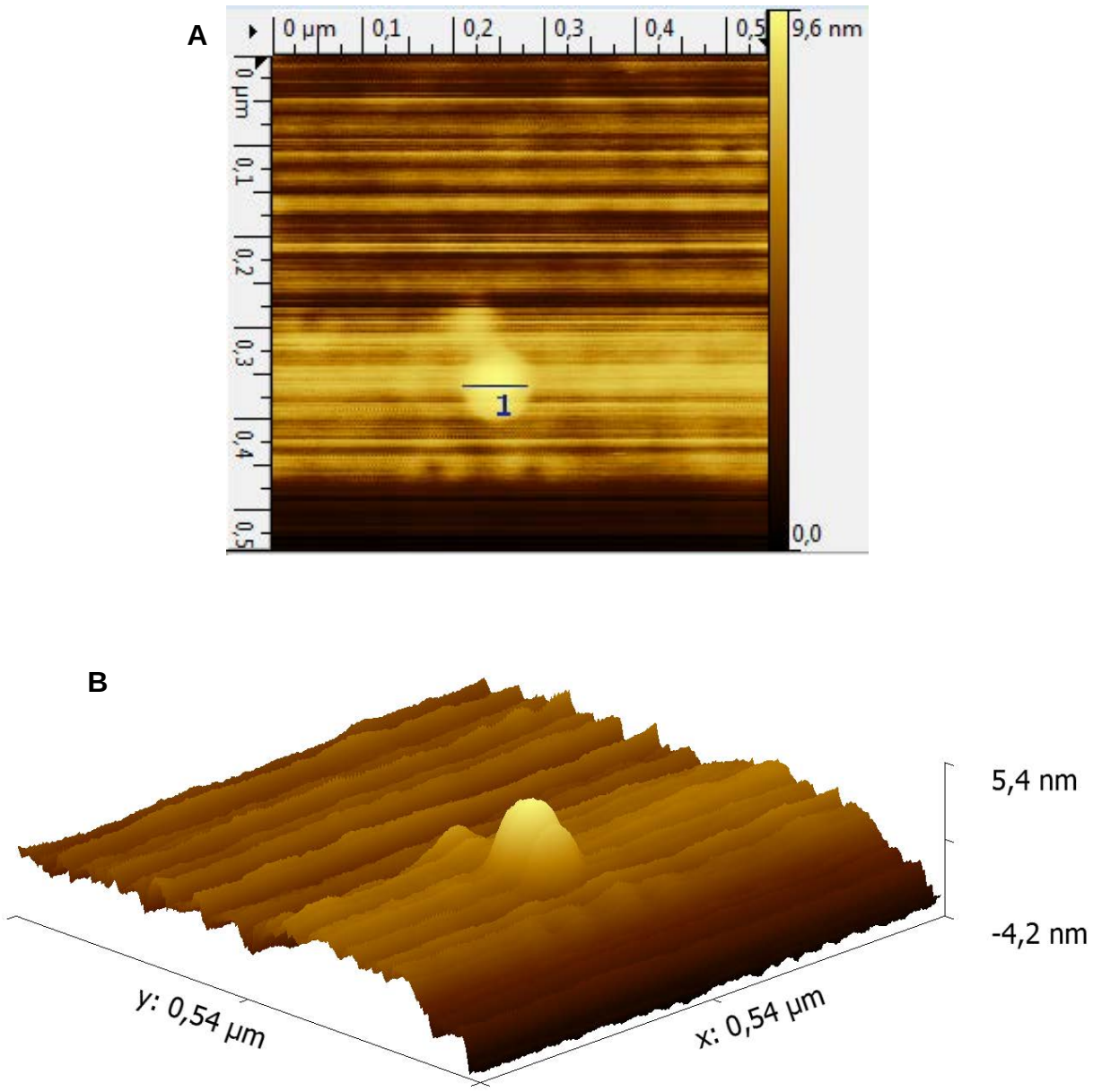


Fig. 10. AFM images showing **A** giving a diameter of ~ 100 nm and **B** corresponding elevation profile, which shows two particles next to each other and a height of ~ 5.4 nm.

Particle characterisation by Dynamic Light Scattering (DLS)

In order to determine size distribution of suspended particles DLS was performed (Fig. 11).

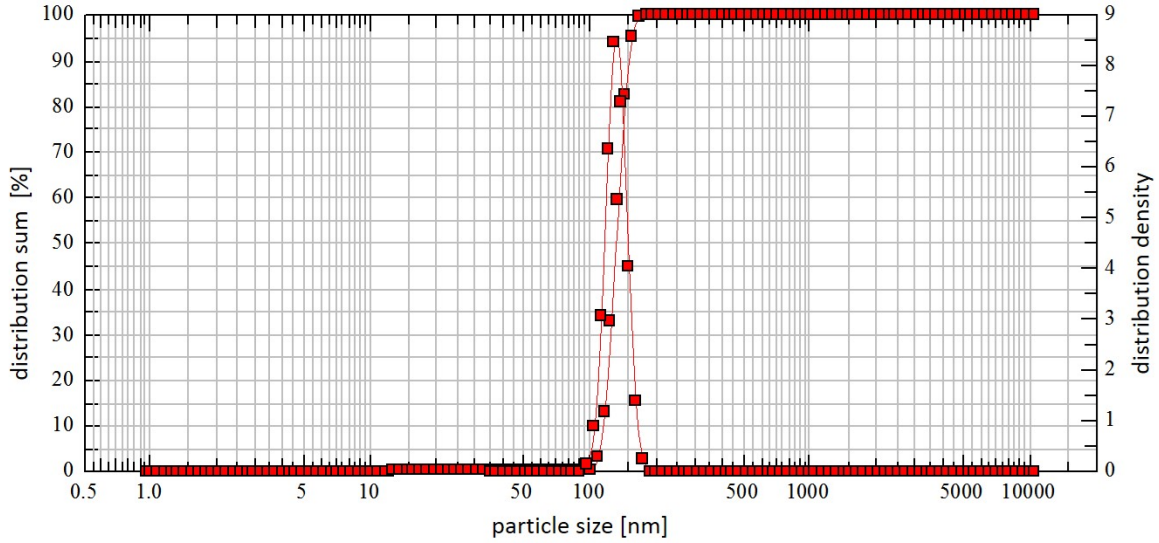


Fig. 11. Typical particle size distribution of DNA loaded crosslinked PS nanoparticles measured using dynamic light scattering. Particles have a size of ~ 150 nm.

DLS gives a hydrodynamic diameter of approximately 150 nm in diameter, while nES GEMMA gave a radius of 53 nm in average and AFM provided a value of approximately 100 nm in diameter. Unlike DLS, nES GEMMA applies a charge to separate particles, which gives the most reliable results. Corresponding to this ~ 53 nm diameter and the fact that AFM detected two agglomerated particles with a size of ~ 100 nm the DLS is detecting at least three particles sticking to each other. Differences should be explained by the fact that DLS is taking place in suspension. These findings indicate that the particle suspension is not as stable as desired. Without further stabilisation of the particle suspension by the addition of surfactants, particles tend to flocculate. Moreover, particles in suspension are moving and rotating which means you get the widest diameter possible. For instance three particles aggregated, could look like a bar, a corner or a triangle (Fig. 12).

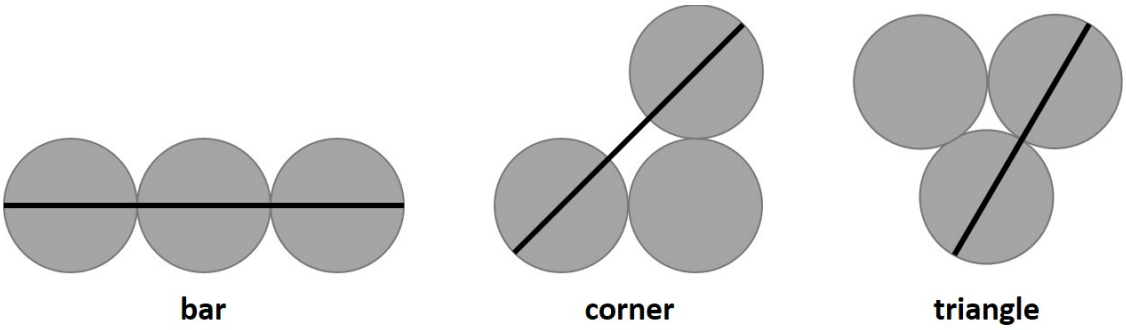


Fig. 12. Different shapes formed by aggregation of three particles with decreasing diameter from left to the right.

Moreover, there is the possibility that of even more than three particles flocculated could show a similar diameter, e.g. five particles forming a flat cross or even seven particles forming a six-armed cross.

3.3 DNA Encapsulation Efficiency

In order to detect and quantify the DNA released from the particles by RaNi mediated cleavage of the disulphide bond of the crosslinker, a more sophisticated method was required, namely Real-time Polymerase Chain Reaction (qPCR), which allows detection of DNA to very low concentrations (10^{-6} ng per reaction volume, typically 2 µl) by comparing to a standard calibration curve. This means, assuming 100% encapsulation efficiency we need to collect 10^6 particles (ppb) from the 10^{15} particles synthesised in a typical batch (~ 9 x 10^3 ng DNA) for detection and quantification.
To determine a standard calibration curve to compare the samples with, a standard dilution series of known DNA concentrations was prepared. The resulting amplification plot measured for the standard calibration curve and the “no template control’” (NTC) is shown in Fig. 13. Concentrations and Ct mean values of the standards and the NTC are shown in Tab. 4.

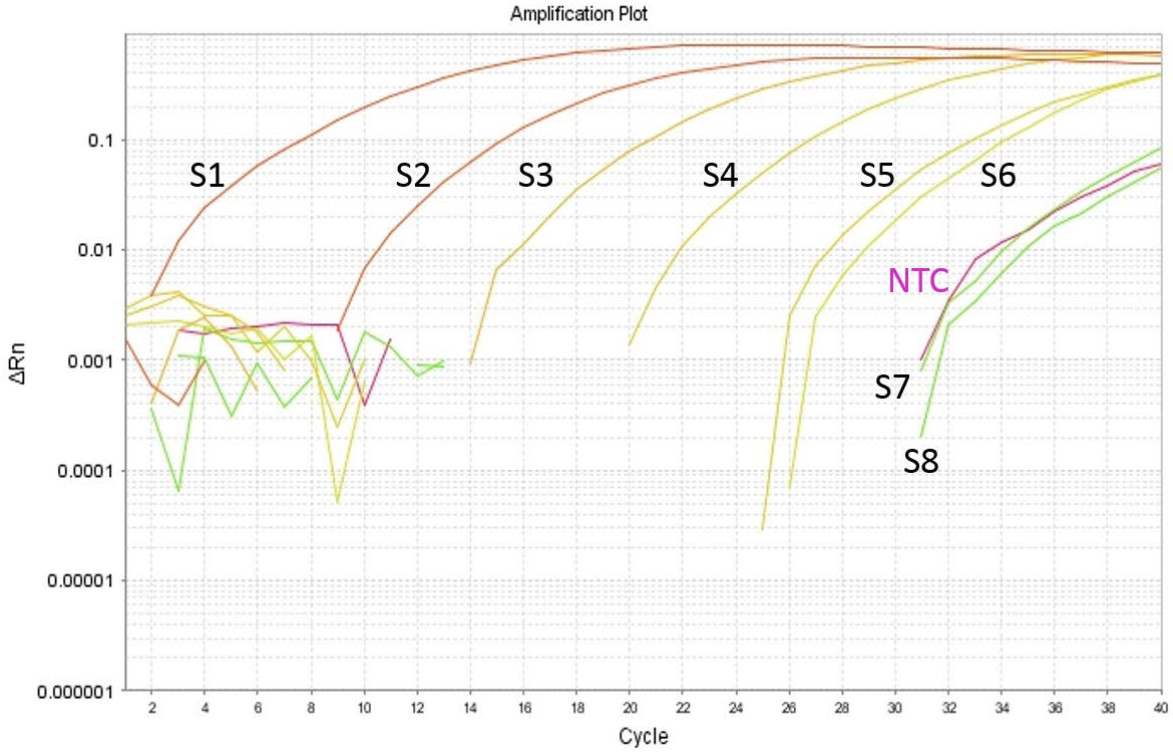


Fig. 13. Typical Amplification plot of standard concentrations (S1 - S8) measured for standard calibration curve and “no template control” (NTC).

Standard	DNA [ng/2 µL]	Ct Mean
S1	$1.80x10^{-1}$	4.240258
S2	$1.80x10^{-2}$	12.04205
S3	$1.80x10^{-3}$	18.02050
S4	$1.80x10^{-4}$	23.59024
S5	$1.80x10^{-5}$	29.33057
S6	$1.80x10^{-6}$	30.68299
S7	$1.80x10^{-7}$	34.41685
S8	$1.80x10^{-8}$	33.40390
no template control (NTC)	0	33.15830

Tab. 4. Ct mean values and DNA concentrations of standards and “no template control” used to determine a standard calibration curve.

Since the Ct mean values of standard 7 and standard 8 are higher than the one of the NTC, the last value used for the standard calibration curve is standard 6. Based on the equation of the standard calibration curve (Fig. 14), the DNA concentrations for the samples and a mass balance have been calculated (Tab. 5).

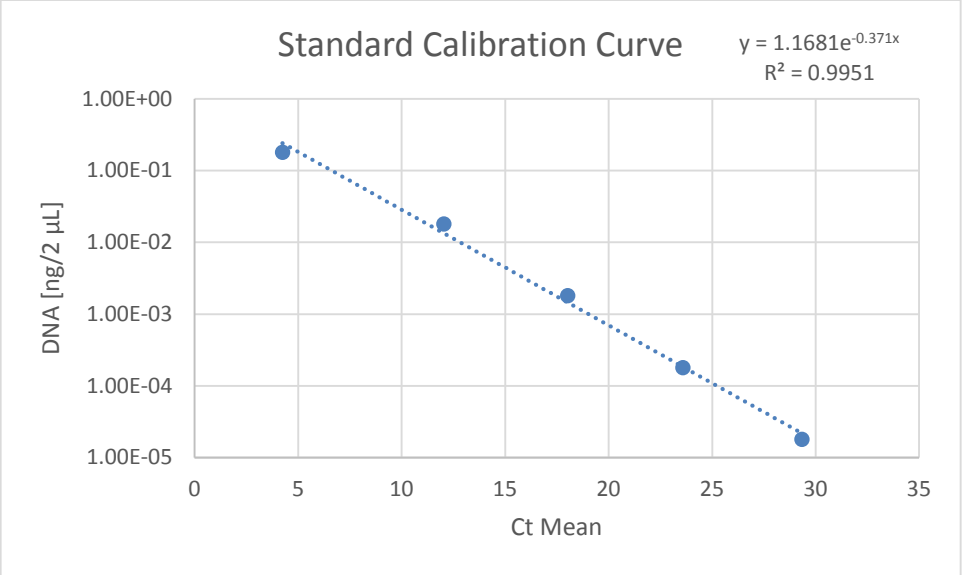


Fig. 14. Standard calibration curve used to calculate DNA encapsulation efficiency

Sample	Ct Mean	DNA [ng/2 µL]	encapsulated DNA [ng]	Output DNA [ng]	Input DNA [ng]
broken particles	21.70971	3.71 x10^{-4}	1.11 x10^{3}	1.11 x10^{3}	9.00 x10^{3}
SDS wash	30.53592	1.40 x10^{-5}		5.38 x10^{-2}	
EtOH wash	31.76634	8.90 x10^{-6}		3.41 x10^{-2}	
			12%	12%	100%

Tab. 5. Ct mean values and amount of recovered DNA after breaking process.

It was possible to recover 12% of DNA of the theoretical amount, which was to be encapsulated into the particles. However, no DNA detected in the washing solutions of particles separated from the suspension by centrifugation, which were subsequently freeze-dried. This indicated that the remaining part of the DNA was complexed with DODAB and the non-complexed DNA remained in the aqueous phase. Furthermore, it is possible that the DNA/DODAB complex remains adsorbed on the surface of RaNi after the breaking process and even after the EtOH washing step. Moreover, the effectiveness of this method depends on the particle size of the RaNi and rarely breaks all particles. Nevertheless, the amount of 12% encapsulated DNA is sufficient to be detected unambiguously and therefore to allow for application in tracer technology.

3.4 Particle Recovery

In order to demonstrate the recovery of particles from highly diluted samples, as present in typical flowback fluid of a fracking process, a dilution series of the particle suspension was prepared. After extracting the particles with toluene and drying in vacuum, the particles were broken with RaNi, DNA/DODAB complex was decomplexed with aqueous SDS solution and the DNA was identified and quantified by comparing the qPCR results to a standard calibration curve (Fig.15).

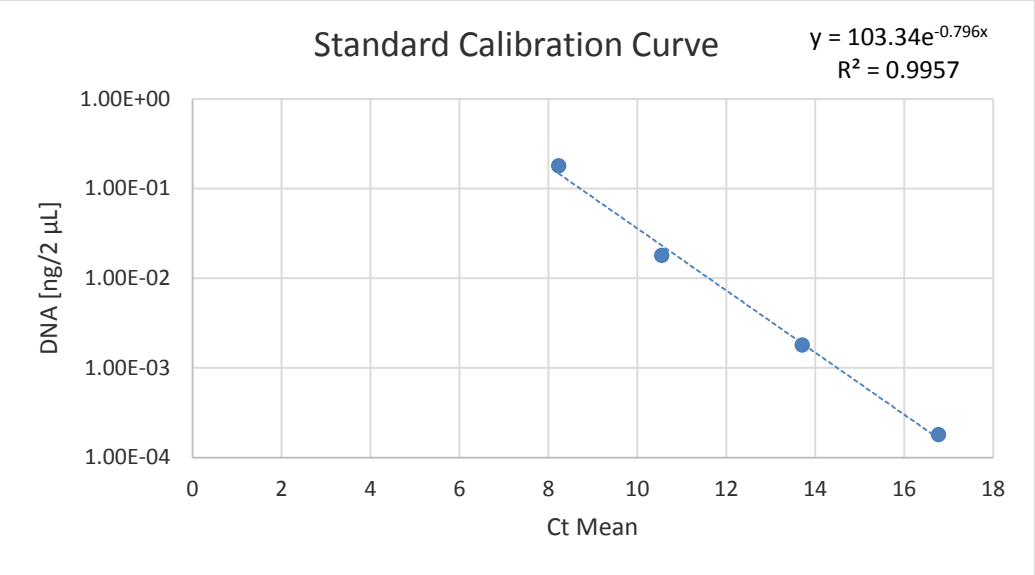


Fig. 15. Standard calibration curve used to calculate the amount of recovered DNA

Standard	DNA [ng/2 µL]	Ct Mean
S1	$1.80x10^{-1}$	8.22712
S2	$1.80x10^{-2}$	10.54956
S3	$1.80x10^{-3}$	13.7036
S4	$1.80x10^{-4}$	16.77054
S5	$1.80x10^{-5}$	17.65989
S6	$1.80x10^{-6}$	17.55343
S7	$1.80x10^{-7}$	16.95504
S8	$1.80x10^{-8}$	17.37426
no template control (NTC)	0	19.41844

Tab. 6. Ct mean values and DNA concentrations of standards and “no template control” used to determine a standard calibration curve.

In this case, standard 1 to standard 4 were used for the standard calibration curve, because due to the low concentration of DNA from standard 5 onwards the results

were drifting off (Tab. 6). This finding should be explained by the detection limits of the qPCR device, namely 10^{-6} ng per measurement.

Sample	Ct Mean	DNA [ng/2 µL]	Output DNA [ng]	Input DNA [ng]
A (10^{14} particles)	15.62811	4.09E-04	1.64E-02	9.00 x10^{2}
B (10^{12} particles)	16.39977	2.21E-04	8.85E-03	9.00 x10^{0}
C (10^{11} particles)	16.45872	2.11E-04	8.44E-03	9.00 x10^{-1}
D (10^{10} particles)	16.71381	1.72E-04	6.89E-03	9.00 x10^{-2}
E (10^{09} particles)	16.68458	1.76E-04	7.05E-03	9.00 x10^{-3}
F (10^{08} particles)	16.64738	1.82E-04	7.27E-03	9.00 x10^{-4}

Tab. 7. Ct mean values and amount of recovered DNA after breaking process.

The amount of recovered DNA appeared to be in the same range throughout the dilution series, which did not correlate to the theoretical input (Tab. 7). This finding indicates that the particles in the suspension are not well dispersed, not even after vigorous shaking, which means that further dilutions would not contain the expected number of particles. That fact confirmed the findings made by DLS analysis of the hydrodynamic diameter in suspension. Another explanation could be the adsorption of the DNA/DODAB complex on the surface of the RaNi and due to the fact, that the same amount of RaNi was used for all the samples and different amount of particles, respectively.

However, it was shown that it is possible to concentrate and collect, respectively, particles out of a highly diluted suspension, break them and detect the DNA.

4. Conclusions

We developed a reproducible in situ polymerisation process to synthesise 100b ssDNA loaded polystyrene nanoparticles crosslinked with a cleavable crosslinker of dimensions constantly smaller than 100 nm in diameter.

By analysing the emulsion prior to the polymerisation process, it was shown that ultrasonication resulted in the formation of a miniemulsion. Furthermore, it was demonstrated that it is possible to size individualised polymerised nanoparticles using nES GEMMA and to image them *via* AFM.

We showed that the DNA could be complexed with a suitable surfactant, such as DODAB, which allowed the DNA to be protected against the elevated temperatures and to be transferred into the monomer phase for encapsulation during the polymerisation process. The encapsulated DNA/DODAB complex could be recovered from the crosslinked nanoparticles by cleaving the disulphide bond of the crosslinker using RaNi mediated reduction, even without an external hydrogen source. The freshly prepared catalyst contained enough hydrogen adsorbed on its surface and could be stored in ethanol for at least one week without losing significant activity. Furthermore, it was shown that it is possible to recover, qualify and quantify free DNA by SDS treatment of the DODAB/DNA complex followed by qPCR analysis.

Determination of the hydrodynamic particle diameter using DLS and qPCR analysis of the dilution series of suspended particles showed that there is a necessity to enhance the colloidal stability of the particle suspension, which strongly depends on the surface chemistry of the crosslinked polymer nanoparticles, i.e. surface charge and/or steric stabilisation imparted by the surfactants used during the emulsion polymerisation. Moreover, the analysis of the dilution series indicated that there is a need to optimise the RaNi breaking process with regard to the ration of RaNito particles that should be broken. Determined droplet size distribution of the emulsion was also indicating the need to further stabilise the suspension and the emulsion respectively.

5. Recommendations for Future Work

One possibility to enhance the stability of the particle suspension is to modify the surface chemistry of the synthesised particles by changing the type of surfactant used to stabilise the miniemulsion prior to polymerisation, or by copolymerisation of, say styrene, with polymerisable surfactants or charged monomers.

Due to the fact, that the recovered amount of DNA from highly diluted suspensions and that it was not possible to recover the entire DNA that was supposed to be encapsulated, it is necessary to evaluate the steps in which the DNA could be lost or destroyed in more detail. Therefor the RaNi breaking process and the DODAB complexation process require further investigation and optimisation. Furthermore, it would be beneficial to identify alternative breaking methods using chemical reducing agents, such as $NaBH_4$, $LiAlH_4$, B_2H_6 or 2-mercaptoethanol to enable to break all particles (of all sizes) to release intact DNA. An effective and reproducible breaking method to recover intact DNA is needed to perform a full mass balance for DNA to determine the encapsulation efficiency.

Another important process that needs to be investigated in more detail is the complexation process itself. In order to ensure all synthetic DNA is sufficiently complexed and rendered hydrophobic to be transferred from the aqueous DNA/TE-buffer into styrene, various surfactants and the complexation equilibrium and kinetics need to be investigated.

For preliminary studies, DODAB was chosen. However, other double-chained cationic (*N*-alkyl-*N*-alkyl′-*N*,*N*-dimethylammonium bromide) surfactants might be suitable both to protect and hydrophobise the DNA.

6. Abbreviations

100b	100 bases
20b	20 bases
B_2H_6	boron hydride
cPS	crosslinked polystyrene
DLS	Dynamic Light Scattering
DODAB	dimethyldioctadecylammonium bromide
DODAC	dimethyldioctadecylammonium chloride
DSDMA	bis(2-methycryloyl)oxyethyl disulphide
dsDNA	double stranded DNA
DTT	dithiothreitol
GE	Gel Electrophoresis
kbp	kilobasepair
$LiAlH_4$	lithium aluminium hydride
LP	left primer
mD	milliDarcy
$NaBH_4$	sodium borohydride
nES GEMMA	Nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analysis
NiAl	nickel aluminide
NTC	no template control
PCR	Polymerase Chain Reaction
PLA	polylactic acid
PS	polystyrene
PS-it	isotactic polystyrene
qPCR	Real-time Polymerase Chain Reaction
RaNi	Raney Nickel
SDS	sodium dodecyl sulphate
SEM	Scanning Electron Microscopy
S-S	disulphide bond

ssDNA	single stranded DNA
TCEP	tris(2-carboxyethyl)phosphine
T_g	glass transition temperature
T_m	melting temperature
V-50	2,2’-Azobis(2-methylpropionamidine) dihydrochloride

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8. English Abstract

There is a great demand for unambiguous, robust tracer systems in oil and gas industry. Tracers that can be added into injection fluids and survive the typical reservoir conditions could picture the connectivity of drilling sites and fracture networks. An unambiguous marked tracer would constitute a simple solution to solve conflicts in water contamination cases, as it can be used to map fluid flow and fluid distribution in geological reservoirs or to trace wastewater effluent leakage from landfill sites or contaminated surface water.

Numerous nonreactive easily differentiated material, dye, chemical or isotope markers are already used. All of these tracer compounds can be harmful to the operators and/or the environment and can easily be adulterated. Moreover, they do not allow not tracking the movement of mixing of fluids stemming from multiple sources. To address these deficiencies a robust DNA tracer system was developed. Uniquely coded synthetic 100 base single stranded DNA (100b ssDNA) is used as a marker. Since DNA is a labile water-soluble polymer, it needs to be protected against environmental influences. Therefore, the DNA is encapsulated into polystyrene nanoparticles crosslinked with a labile disulphite crosslinker, which are synthesised by polymerisation of miniemulsions stabilised by surfactants. Protection, hydrophobisation and transfer of the DNA into the monomer phase is ensured by complexation of the DNA with cationic dialkyl amphiphiles. This allows the DNA to be more resistant to elevated temperatures and pH and for effective transfer into the organic monomer phase. Once the particles are collected from the flowback fluid, intact DNA needs to be recovered. By taking advantage of the disulphide containing dimethycrylate used to crosslink the polystyrene the particles can be “broken”. The breaking method of choice is metal mediated reduction with hydrogen using Raney Nickel (RaNi). In order to extract free, detectable ssDNA, the DNA/cationic surfactant complex is decomplexed using an anionic surfactant. Identification, quantification and determination of DNA encapsulation efficiency is done by real-time polymerase chain reaction (qPCR). The particles are characterised using scanning electron microscopy (SEM), Dynamic Light Scattering

(DLS) and Nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analysis (nES GEMMA). It was shown that complexation and encapsulation of DNA into polymer nanoparticles and recovery and concentration thereof by use of emulsification is working. Furthermore, breaking of these particles using RaNi and detection and quantification of released DNA *via* qPCR was demonstrated.

9. German Abstract

Von Seiten der Öl- und Gasindustrie und Umweltschutzorganisationen herrscht eine große Nachfrage nach eindeutigen, robusten Tracer-Systemen. Da es ein solcher Tracer, der der Injektionsflüssigkeit zugesetzt werden kann und die typischen Reservoirbedingungen intakt übersteht, ermöglichen würde, Flüssigkeitsbewegungen und deren Flüssigkeitsverteilung in geologischen Reservoiren zu verfolgen, könnte er darüber hinaus Aufschluss über die Verbindung von Bohrlöchern an verschiedenen Standorten und über Frackingnetzwerke geben. Außerdem sich daraus eine einfache und schnelle Lösung zur Aufklärung von Kontaminationsfällen von Grundwasser durch Sickerwasser von Mülldeponien oder Abwasser ergeben.

Es finden bereits viele verschiedene unreaktive, einfach unterscheidbare chemische Tracersysteme, wie z.B. Farbstoffe, Chemikalien- oder Isotopenmarker Anwendung. Der Nachteil derer besteht allerdings darin, dass diese schädlich für Anwender und/oder die Umwelt sein können. Darüberhinaus kann mit Hilfe dieser Tracer keine Aussage über vermischte Prozessflüssigkeiten getroffen werden. Um genau diese Untersuchungen zu ermöglichen, wurde ein robustes DNA-Tracer-System entwickelt. Als Marker hierbei dient eine 100 Basen lange, einzigartig kodierte, einsträngige DNA (100b ssDNA). Da allerdings DNA ein labiles, wasserlösliches Polymer ist, muss sie gegen Umwelteinflüsse geschützt werden. Daher wurde die DNA in durch Polymerisation einer Tensid-stabilisierten Miniemulsion hergestellte quervernetzte Polystyrolnanopartikel eingeschlossen. Der Schutz, die Hydrophobisierung und der Transport in die Monomerphase wurde durch Komplexierung der DNA mit einem kationischen Dialkylamphiphil sichergestellt. Diese Komplexierung bedingt die Widerstandsfähigkeit der DNA gegenüber höheren Temperaturen und pH-Werten. Nach Trennung der Tracer-Partikel von der rückströmenden Flüssigkeit, muss intakte DNA zurückgewonnen werden. Dazu wurden die Polystyrolpartikel mit Hilfe eines Disulfidbrücken-enthaltenden Dimethacrylats quervernetzt. Diese Disulfidbrücken können über Raney Nickel (RaNi) katalysierte Reaktion gebrochen werden. Anschließend wurde der

freigesetzte DNA Komplex durch Behandlung mit einem anionischen Tensid dekomplexiert und mittels Echtzeit-Polymerase-Kettenreaktion (qPCR) identifiziert und quantifiziert. Die Partikel wurden via Rasterelektronenmikroskop (SEM), Dynamische Lichtstreuung (DLS) und Nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analysis (nES GEMMA) charakterisiert. Es wurde gezeigt, dass die Komplexierung und der Einschluss von DNA in Polymernanopartikel und deren Rückgewinnung und Aufkonzentration mittels Emulgierung funktioniert. Außerdem wurde das Brechen der Partikel mit Hilfe von RaNi und die Qualifizierung und Quantifizierung der dabei freigesetzten DNA *via* qPCR demonstriert.

10. Curriculum Vitae

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