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Microcapsules encapsulating a nucleic acid amplification reaction mixture and their use as reaction compartments for parallel reactions

Description

The present invention refers to microcapsules of controlled permeability encapsulating a nucleic acid amplification reaction mixture, in particular, a PCR reaction mixture and providing a compartment to perform nucleic acid polymerization and/or amplification, in particular, a PCR reaction. During the nucleic acid polymerization and/or amplification, in particular, the PCR reaction, high molecular weight nucleic acid product, in particular, a PCR product is accumulated in the interior of capsules that contain polymerase and appropriate templates and primers, while low molecular weight substrate molecules, in particular dNTPs and/or ddNTPs, permeate into the capsule from the outside during the reaction. The invention also describes the encapsulation of a nucleic acid amplification reaction mixture, in particular, a PCR reaction mixture into microcapsules and the use of such capsules to carry out multiple nucleic acid polymerizations and/or amplifications, in particular, PCR reactions simultaneously. The described capsules create a compartment that separates individual nucleic acid polymerization and/or amplification reactions, in particular, PCR reactions. Furthermore the invention describes the detection, isolation and analysis of capsules that contain a nucleic acid product, in particular, a PCR product. Application examples of simultaneous multiple nucleic acid polymerizations and/or amplifications, in particular, PCR for analytical purpose are provided.

In the state of the art PCR reactions are carried out in reaction tubes or micro titer plates or in glass capillaries. The reaction volume varies from 50 to 200 µl for tubes to 10 to 100 µl for microtiter plates and below to 20 µl

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for glass capillaries used by the latest and most advanced PCR cycler from Roche (LightCycler). The parallelism of PCR is restricted to about 48 reactions by using tubes and 96 to less than 2000 reactions by using high-density microtiter plates. Instruments using glass capillaries can perform 96 reactions in parallel. This numbers are still too small to perform screening projects, e.g. in pharmacological research in a appropriate time. The advantage of the latest PCR cycler models (e.g. Roche LightCycler) is that they are capable to perform Real-time PCR. In Real-time PCR, information about the generation of PCR products and sequence information can be obtained for individual reactions in real-time. In current PCR technique, individual PCR reactions are separated by using tubes, capillaries or wells. These tubes capillaries or wells are made from plastic ware (PS or PP) or glass. To perform a higher number of reactions in parallel, the integration density must be increased and the reaction volume must be decreased. To increase the number of parallel PCR reactions with current technology has limited chance to succeed. The standard microtiter plate format is already at the edge of its integration with 1539 cavities per plate. Furthermore, the filling of this large number of cavities becomes a substantial problem.

By using state of the art PCR equipment about 48 to 1539 PCR reactions are carried out per run in parallel. Special instruments can handle more than one PCR microtiter plate to increase the throughput. However, the increase is only about one order of magnitude and the instruments need big lab space.

T. Oberholzer et al., Chemistry & biology, 2 (1995), 677-682 describe a polymerase chain reaction in liposomes. However, liposomes are unpermeable to the components of a PCR reaction so that all components must initially be present within the liposomes thus greatly limiting the practical application.

Therefore, it was an object of the present invention to provide an improved nucleic acid amplification process. In particular, it was an object of the present invention to provide a nucleic acid amplification process which allows for the parallel performance of a large number of parallel nucleic acid amplification reactions.

This object is achieved according to the invention by a nucleic acid amplification process comprising the steps

(a) providing a nucleic acid amplification reaction mixture,

(b) encapsulating said reaction mixture into capsules being permeable for low molecular weight molecules and not permeable for high molecular weight molecules and thereby forming capsules comprising constituents of the reaction mixture, and

(c) performing at least one amplification step and thereby amplifying a nucleic acid within one or more of the capsules.

The present invention describes a method which allows carrying out up to 10^5 to 10^{11} nucleic acid amplifications, in particular, PCR reactions in parallel.

The present invention is based on the encapsulation of a nucleic acid amplification reaction mixture, in particular, a PCR reaction mixture into microcapsules with high permeability for low molecular weight molecules and no permeability for high molecular weight molecules. The capsules create a compartment to separate individual nucleic acid amplifications, in particular, PCR reactions from each other. During the nucleic acid amplification, high molecular weight nucleic acid amplification product, in particular, a PCR product is accumulated in the interior of capsules that contain polymerase and appropriate templates and primers, low molecular weight substrate molecules, in particular dNTPs, permeate into the capsule from the outside. Low molecular weight molecules according to the invention have a molecular weight of less than 2000 Da, in particular less

than 1500 Da and preferably less than 1200 Da and comprise e.g. dNTPs, ddNTPs and labeled, in particular fluorescent labeled nucleotides. High molecular weight molecules have accordingly a molecular weight of greater than 2000 Da, in particular greater than 2500 Da and preferably greater than 3000 Da and comprise e.g. polymerase, primers, templates and amplification products.

Typical capsule diameters are in the range of 50 nm to 1000 μm, preferably 500 nm to 100 μm, and most preferably 40 to 60 μm. Capsules are preferably dispersed in a buffer, e.g. a PCR buffer (containing dNTPs) with a density of about 105 to 1011 capsules per ml. By using microcapsules encapsulating a nucleic acid amplification reaction mixture, in particular, a PCR reaction mixture a very large number of parallel reactions in a small volume is possible. In comparison to prior art technology a microcapsule is used to separate individual nucleic acid amplifications, in particular, PCR reactions and not a tube or well made from plastic ware or glass. By using the present invention a number of up to about 105 to 1011 parallel nucleic acid amplifications, in particular, PCR reactions is possible (depending on capsule size and density) in a single nucleic acid amplification, in particular, a PCR run. A preferred application is the isolation of single DNA strands from a complex DNA mixture, as well as screening, sequencing and construction of DNA libraries. Flow cytometry is a powerful method to analyze and separate capsules that contain nucleic acid amplification product, in particular, a PCR product. The present invention provides a method for mass screening with a throughput that is not realizable with prior art techniques.

The present invention refers to microcapsules encapsulating a nucleic acid amplification reaction mixture, in particular, a PCR reaction mixture and providing a compartment to perform a nucleic acid amplification, in particular, a PCR reaction. In a first step, a nucleic acid amplification mixture is provided. This mixture may contain all or some of the reaction

partners or components required for performing a nucleic acid amplification mixture. Additional components may be added after capsule formation. Preferably, a PCR reaction mixture consisting of: polymerase and/or primers and/or templates and/or buffer substances and/or dNTPs and/or enhancers and/or DNA probes are provided and, more preferably, mixed with a microparticle-forming matrix material (e.g., agarobe) and microparticles of the matrix material with incorporated PCR reaction mixture are formed (e.g., by ionotropic gel formation of alginate or agarose) (Figure 1/2 3). An alternative method is the incorporation of the reaction mixture into a porous microparticle as a matrix. A detailed description of the method is given in the section EXAMPLES.

In a second step, the nucleic acid amplification mixture is encapsulated. In this step capsules comprising reaction partners or constituents of the reaction mixture are formed. While the reaction mixture itself can be encapsulated, it is preferred to encapsulate matrix particles containing the reaction mixture. In this embodiment the constituents of the nucleic acid amplification reaction mixture are incorporated into a matrix material prior to encapsulation. Suitable matrix materials are, for example, porous microparticles, wax-like substances, agarose, alginates, polysaccharides, a polypeptide, fatty acids, salts of fatty acids, polyvinylpyrrolidone (PVP) or the matrix is ice, or mixtures thereof. In a preferred embodiment, matrix material (e.g., agarose, alginate, microparticle) with incorporated PCR reaction mixture is subsequently encapsulated (Figure 1/2 4). A variety of methods can be used for encapsulation, e.g. Layer-by-Layer technology or interfacial polymerization or cavaciation. The permeability of the capsule can be controlled in a way that small molecules (e.g., dNTPs, ddNTPs, short PCR primers) can permeate through the capsule wall but larger molecules (e.g., DNA templates, large PCR primers, PCR products, polymerase) cannot pass the capsule.

A requirement for using the Layer-by-Layer technology for encapsulation is a charged template (the material to be encapsulated). Agarose and alginate microparticles are negatively charged and a suitable template. Porous microparticles are available with negative or positive surface charges. By using the Layer-by-Layer encapsulation technique a permeability control is achieved by controlling the number of polyelectrolyte layers, the polyelectrolyte material, the molecular weight of the polyelectrolyte and the crosslinking.

Optionally, the matrix material is dissolved or removed out of the capsule (Figure 1/2 5) after the encapsulation process is performed. E.g., for alginate-based matrix material by an exchange to a calcium free buffer. Dissolving or removing of the matrix material can be performed, e.g. by dissolution with solvents, by chemical means such as oxidation, or by high temperatures during PCR cycles. Agarose matrix material will melt under PCR temperature cycling conditions resulting in high permeability of molecules within the capsule.

Next, at least one amplification step, e.g. PCR cycling, is performed to amplify a nucleic acid within one or more of the capsules. Prior to the amplification step preferably non-encapsulated reaction mixture compounds are separated from the capsules, e.g. by centrifugation, filtration, dialysis, or destruction with nucleases and/or proteases. The dispersion of microcapsules in buffer, e.g. a PCR buffer is used for nucleic acid amplification, in particular, PCR experiments. PCR cycling is achieved by placing a reaction tube (or micro titer plate) containing microcapsules dispersion in a conventional PCR cycler programmed with standard PCR cycling temperature profiles.

A particular advantage of the process according to the invention is that components, constituents or reactants of the nucleic acid amplification reaction can be introduced selective into the capsules during and/or before

the amplification and/or polymerization reaction is performed in a continuous or batchwise manner. Preferably, the permeability of the capsules is controlled to allow selective diffusion of starting materials into the capsules such as dNTPs but restrict the out-diffusion of reaction products or templates. This allows for a continuous or batchwise supplementation of one or several starting materials into the capsules and for further enrichment of the desired product. Thus, the performance is not limited to initially present reactants.

For example, continuous selective exchange can take place during amplification. Layer-by-Layer encapsulated nucleic acid amplification reaction mixtures, in particular, PCR mixtures can exchange low molecular reactants, in particular dNTPs, with the extra capsular media. The dNTPs can easily diffuse through the capsule wall. Thus, dNTPs can be added to the reaction mixture. The optimal dNTP concentration can be kept constant in the interior of the capsule over a long period of time. The nucleic acid amplification or polymerization, in particular, a PCR reaction is therefore not limited to the building block (e.g. dNTPs) material in the capsule.

If preferably fluorescent labeled dNTPs are used, amplification products are labeled and capsules containing amplification products can be easily identified. If 4 color fluorescent labeled ddNTPs are used, polymerization producta are fluorescent labeled regarding to their 3'-end base.

For example, batchwise selective exchange can take place before or during amplification. Constituents, components or reactants can be transferred into the capsule by a triggered change in capsule wall permeability. The capsule wall permeability, for example, can be varied or changed by a pH change, e.g. pH dependent swelling or shrinking of hydrogel capsule materials, or an ionic strength change of the reaction medium. In a more preferred process, capsules are constructed of 3 to 6 layers of thiolated polyelectrolytes in a Layer-by-Layer approach with an outer non-thiolated

layer. In the presence of oxygen, thiol groups form disulfide bridges leading to a crosslinking of polyelectrolyte layers resulting in lower permeability. Addition of reductive agent (e.g. dithiotritol, DTT) results in a reduction of disulfide groups to thiols and reverses the process, resulting in higher capsule permeability. In addition, this principle can be applied to create stable crosslinked capsule to perform PCR cycling and to intentionally break such capsules e.g., to release DNA products to perform electrophoresis experiments.

Continuous selective exchange during amplification and batchwise addition of reactants can be combined e.g. first a DNA sequence is amplified by PCR using continuous inflow of dNTPs followed by batchwise addition of fluorescent labeled ddNTPs to generate 3'-end fluorescent labeled products.

Preferably, the process according to the invention comprises the further step

(d) detecting capsules containing a nucleic acid product. This step can be performed e.g. by using flow cytometry or microscopy.

In a further preferred embodiment, one or more components of the nucleic acid amplification reaction mixture and/or of the capsule material carry a label. Suitable labels are e.g. fluorophores, quantum dots, radioisotopes, dyes or NMR active isotopes or nanoparticles. In particular, the labels are selected to enable the encoding of individual capsules and/or the encoding of distinct reaction products.

Positive capsules, i.e. capsules containing nucleic acid product, are preferably separated from the reaction mixture. For example, capsules containing nucleic acid products can be detected by the physical signature caused by a label or by dyes staining DNA or by UV adsorption measurement of DNA and are separated and isolated from the capsule

suspension. The nucleic acid product of said capsules can then be analyzed or extracted for further use. Preferably a PCR product is extracted from separated capsules or a single capsule.

Oberholzer et al. investigated in the origin of live and performed a PCR reaction in liposomes (Chemistry & Biology, 2:677-682, 1995). Liposome encapsulated nucleic acid amplification reaction mixtures however, cannot exchange DNA, primer, staining dyes or dNTP material with the external media. The lipid bilayers of liposomes do not allow diffusion of charged molecules (e.g. all PCR mixture compounds are charged and/or macromolecular). Therefore the PCR reaction comes to still stand after most of the dNTPs are used up. Also capsules with PCR product cannot be distinguished from capsules without PCR product.

In the current invention preferably labeled starting materials, e.g. labeled dNTPs or labeled ddNTPS are used resulting in labeled nucleic acids, e.g. in a fluorescent labeled PCR product, which is accumulated in capsules with PCR reaction. Such capsules can be easily detected e.g. due to their fluorescent intensity, and isolated, e.g. by UV or fluorescent detection.

In a preferred embodiment, the PCR reaction takes only place in capsules that contain a template and a complementary pair of primers (Figure 2/2 2 and 2b). Other capsules (Figure 2/2 1 and 3) will not build PCR product. By using fluorescent-labeled dNTPs in the PCR experiment, PCR product is labeled with fluorescent molecules. Fluorescence intensity increases for capsules with PCR reaction ("positive capsules") during the PCR cycling. An alternative way is the use of intercalating reporter dyes. These dyes (e.g., ethidium bromide, SYBR Green I) increase in fluorescent light intensity while intercalating with double stranded DNA (= PCR product). Sequence information of nucleic acid amplification products, in particular, PCR products can be obtained by using hybridization probes (e.g., Tag Man system or molecular beacons).

Positive capsules can be detected by flow cytometry and separated from the bulk by flow sorting. Modern flow cytometry instruments are capable of analyzing 70 000 particles per second with high accuracy. A microcapsule suspension with 109 capsules needs an analyzing time of about 4 hours. Modern instruments are equipped with multicolor analysis. This method can be used to identify encoded capsules or to track single capsules in complex mixtures. By using flow cytometry the present invention provides a method for mass screening with extremely high throughput.

In another embodiment of the invention, microcapsules encapsulating a nucleic acid amplification reaction mixture, in particular, a PCR reaction mixture are fixed onto a 2-dimensional surface. The location of individual capsules is determined by a video camera. A laser that illuminates the fixed capsules introduces energy to heat up individual capsules to a desired PCR cycling temperature. The laser scans over the surface (e.g., by using electro movable mirrors and optic) and addresses single capsules or a certain surface area. Different PCR temperature profiles are applicable for single capsules or a certain surface area. The capsule temperature is controllable by the laser power and the illumination time. Laser power adsorption can be increased by dyes incorporated into the capsule wall. In addition dyes can be used to locate capsule positions. By using fluorescent labeled dNTPs the increase of PCR product in capsules is online measurable by an increase in fluorescent light intensity originated from capsules containing PCR product. This allows real time PCR of individual capsules. Optimal PCR temperature profiles for individual capsules can be evolved by the sequential application of different PCR temperature profiles and real-time measurement of fluorescent intensity increase. By using an intelligent feedback loop temperature profiles can be optimized and evolved for maximum amplification or specificity.

Most preferred in the process according to the invention, the nucleic acid amplification reaction is a polymerase chain reaction (PCR). In this case the process preferably comprises the steps:

(i) providing a PCR reaction mixture consisting of polymerase and/or dNTPs and/or buffer substances and/or enhancers and/or primers and/or templates and/or DNA probes and/or matrix material,

(ii) encapsulating said PCR reaction mixture in capsules being permeable for low molecular weight molecules and not permeable for high molecular weight molecules and thereby forming capsules comprising constituents of the reaction mixture and

(iii) performing at least one amplification step comprising PCR temperature cycling and thereby amplifying a nucleic acid within one or more of the capsules.

The present invention further relates to a capsule encapsulating a nucleic acid polymerizations and/or amplification reaction mixture, in particular, a PCR reaction mixture and/or a nucleic acid amplification product, in particular, a PCR product. These capsules can be advantageously used for a variety of applications. Besides or after the use as reaction compartment for parallel nucleic acid amplification and/or polymerization reactions they can be applied for the analysis and/or screening of nucleic acids or artificial derivatives of nucleic acids, for the production of nucleic acid libraries, for nucleic acid sequencing, in bioassays, or in real-time PCR.

In another embodiment of the invention, microcapsules encapsulating a nucleic acid amplification reaction mixture, in particular, a PCR reaction mixture are used to construct a genomic DNA library comprising the steps:

(i) isolation of genomic DNA,

(ii) partial digestion of the genomic DNA with restriction enzymes into fragments of 500 to 50 kb having sticky ends (e.g. with Sau3A),

(iii) extending both ends of the genomic DNA fragments with a known DNA by hybridization and ligation to create recombinant DNA, (e.g.

by using λ phage DNA treated with BamHI nuclease to create sticky ends complementary to the genomic DNA fragments. Removal of the out cut middle part (=replaceable region) of λ phage DNA. Hybridization of λ phage DNA fragments with genomic DNA fragments. Ligation of fragments creates recombinant DNA).

(iv) preparation of a PCR reaction mixture with recombinant DNA as a template and a primer pair complementary to a region that amplify the genomic DNA fragment,

(v) encapsulation of said mixture into microcapsules, resulting in capsules carrying statistically one copy per capsule and

(vi) performing nucleic acid amplification.

In another embodiment of the invention, microcapsules encapsulating a nucleic acid amplification reaction mixture, in particular, a PCR reaction mixture are used to construct a cDNA library comprising the steps:

(i) isolation of mRNA,

(ii) hybridization of poly(T) oligonucleotide onto the mRNA,

(iii) elongation of the poly (T) primer with reverse transcriptase,

(iv) hydrolyzation of the mRNA with alkali resulting in single stranded cDNA,

(v) Addition of a oligonucleotide tail, e.g. poly(G), onto the 3' terminus with terminal transferase,

(vi) preparation of a PCR reaction mixture with cDNA as a template and a primer pair that allows PCR amplification of the coding region, e.g., poly(T) and poly(C),

(vii) encapsulation of said mixture into microcapsules, resulting in capsules carrying statistically one copy cDNA per capsule, and

(viii) performing nucleic acid amplification.

In another embodiment of the invention, the nucleic acid product, in particular a PCR product, is analyzed parallel in multiple capsules by using gel chromatography comprising the steps:

(i) embedding capsules filled with nucleic acid product into a thin gel of agarose or polyacrylamide,

(ii) rendering the capsule wall permeable for the nucleic acid product, by chemical or physical means, resulting in release of the nucleic acids from capsules,

(iii) applying an electrical potential to carry out gel electrophoresis,

(iv) detection of band patterns created from individual capsules, and

(v) computing the lengths of DNA fragments from calibrations obtained with capsules filled with DNA ladder.

In another embodiment of the invention, microcapsules encapsulating a nucleic acid reaction mixture, in particular, a PCR reaction mixture are used to sequence DNA or genomic DNA or cDNA comprising the steps:

(i) performing the steps (i) to (v) described for the construction of DNA libraries or steps (i) to (vii) described for the construction of cDNA libraries, resulting in a preferable length of 500 bp of DNA fragments, but using a primer pair with one primer in excess of another, e.g. at a ratio of 2:1 to 1000:1.

(ii) performing PCR until the primer with the lower concentration is used up.

(iii) adding 4 color fluorescent labeled 2',3'-dideoxynucleoside triphosphates (ddNTPs) in a concentration of about 1/500, e.g. 1/100 to 1/1000, of the dNTP concentration to the suspension of microcapsules and performing elongation of the primer with excess concentration,

(iv) performing the steps (i) to (iii) described for the analysis of nucleic acid capsule content by gel electrophoresis,

(v) detection of the band patterns created from individual capsules, at the wavelengths of the four corresponding dyes, and

(vi) computing the sequence of the DNA fragments and the entire genome.

In addition to the described procedures for the construction of DNA libraries, electrophoresis and sequencing the following modification can be made. For the construction of DNA or genomic libraries: The digestion can be carried out with any nuclease that created sticky ends for extension of the dsDNA with a double stranded DNA tail. E.g., by using BamHI the nuclease cut at 5´-G^GATC-NNN-3´ // 3´-NNN-CTAG^G-5´ and sticky ends of the sequence 5'-GATC-NNN-3' will be formed. Extension can be carried out by hybridization and ligation with dsDNA having a sticky end 5'-GATC-Nn-3' (N = CGAT, n = 1 to 25). This procedure will create identical sequences at the 3'-ends of the DNA molecules. One primer can be used to perform PCR to amplify the two strands.

For the performance of the electrophoretic analysis: The release of nucleic acid product from microcapsules can be achieved e.g., by using disulfide cross linked capsules, described previously. In the gel electrophoresis experiment, each capsule needs at least 50 μm x 5 cm gel area. About 24000 individual electrophoresis experiments can be carried out on an A4 size gel. Representing sequence information of about 10^7 bases. For plasmids, viruses and bacterium chromosome about 1 A4 gel is needed, for the human genome with 3 x 10^9 bp about 250 A4 gels are needed and for one human chromosome about 6 gels are needed. The process according to the invention has the potential to sequence a genome in about 1 day.

For the performance of the sequencing: To sequence a DNA library the DNA fragments are required to have two different sequences at their 3'-end. This can be achieved by using a hybridization/ligation to a plasmid. This allows the hybridization of different primers onto the 3'-ends. To generate fragments with fluorescent labeled 3'-end from only one strand either at the coding or non coding strand, an excess of one primer is used. Therefore, the two primers are added at a ratio of 1:2 to 1:1000. A PCR can be carried out until the primer with the lower concentration is used up. Then, the primer in excess is elongated at the presence of ddNTPs. The

method can be performed with the forward or the backward primer in excess. By carrying out two experiments, one using the forward and the other using the backward primer in excess, two data sets of complementary sequence can be generated. The elongation of the primer in excess can be repeated several times by using a temperature cycling protocol similar to PCR.

Further, the invention comprises a kit for the production of the inventive capsules or the performance of the inventive processes containing at least one starting material necessary for performing the processes.

The invention is further illustrated in the following figures and examples.

FIGURES

Figure 1 shows the preparation of capsules encapsulating a PCR reaction mixture. The reaction mixture compounds (Fig1/2 1) are consisting of dNTPs and/or buffer substances and/or enhancers and/or primers and/or templates and/or polymerase. Prior to their encapsulation the mixture compounds are incorporated into a matrix material (Fig. 1/2 2) resulting in micro particles of matrix material with incorporated reaction mixture (Fig1/2 3). Encapsulation is carried out by using e.g. a layer-by-layer process (Fig. 1/2 4). Subsequently the matrix material is removed leaving the reaction mixture compounds encapsulated into a micro scale capsule (Fig 1/2 5).

Figure 2 shows the application of the encapsulated PCR reaction mixture. The template and/or primers are present in the capsule (Figure 2/2 1-3). A PCR product is generated only in capsules that contain a template complementary to a reverse and forward PCR primer (Figure 2/2 2b). Capsules that contain no template or a template but non-complementary primers cannot generate a PCR product (Figure 2/2 1b and 3b). Capsules

that contain a PCR product are identified e.g. by fluorescent or UV spectroscopy and separated (Figure 2/2 2c). PCR products and templates are extracted from capsules or from a single capsule for further studies (Figure 2/2 2d).

Figure 3 shows that PCR reactions can be successfully performed in agarose gel matrix. Lane: 1 = Ladder (100 bp), 2 = Positive control, 3 = Negative control, 4 = Pos. contr. + 0.1 % agarose, 5 = Pos. contr. + 0.2 % agarose, 6 = Pos. contr. + 0.5 % agarose, 7 = Pos. contr. + 0.6 % agarose, 8 = Pos. contr. + 0.7 % agarose, 9 = Pos. contr. + 0.8 % agarose, 10 = Pos. contr. + 1.0 % agarose, all at a Mg concentration of 1.5 mM.

Figure 4 shows microbeads prepared from agarose (about 50 μm diameter). 4a) Phase contrast micrograph shows the morphology of agarose microbeads. 4b) Fluorescent micrograph (FITC filter) shows the microcapsule of beads prepared with FITC labeled PAH. 4c) Fluorescent micrograph (Ethidium filter) shows ethidium bromide stained dsDNA in the interior of microbeads.

Figure 5 shows the stability of (PAH/PSS)6 encapsulated agarose microbeads (about 50 μm diameter) under PCR temperature cycling conditions (0.5 min at 95 degree, 1 minute at 60 degree and 2 minute at 72 degree). 5a to 5d) micrographs of encapsulated beads after 1, 5, 20 and 30 cycles. 5e) percentage of stable capsules versus PCR cycle number.

EXAMPLES

Materials: Polycation, poly(allylamine hydrochloride) (PAH), Mw15,000, and polyanion, poly(sodium 4-styrenesulfonate) (PSS), Mw 70,000 from

Aldrich. Sodium Alginate from (Manugel) Alginate Industry GmbH. PCR reagents and polymerase from Promega. Templates and primers from Synthetic Genetics.

Example I: Preparation of capsules encapsulating a PCR reaction mixture

A DNA template dilution (in 1 ml 1x PCR buffer) with a density of 103 to 1012 template molecules per ml was prepared. Taq DNA polymerase (25 U/ml), 0.8 μM of each primer (optional, fluorescently labeled) and 200 μg/ml BSA was added to the template solution.

A) By using a porous microparticle: The solution from above was a mixed with a suspension of 103 to 1012 porous micro particles (0.5 to 10 μm in diameter, pore size 5-200 nm) in 100 μl water. The mixture was incubated over night to archive a diffusion of PCR mixture compounds into particle pores (some material may also adsorb onto the particle surface). The particle suspension was centrifuged and the supernatant was discarded. The particles were immediately coated with polyelectrolyte (5 mg PAH in 1 ml 1x PCR buffer). Then the supernatant was removed by centrifugation followed by three cycles of washing with 1xPCR buffer to remove the excess non-adsorbed polyelectrolyte. Then 1 ml of oppositely charged polyelectrolyte solution (5 mg PSS in 1 ml 1x PCR buffer) was added to form a consecutive layer on the particle surface. The centrifugation/washing steps and the consequent adsorption of oppositely charged polyelectrolytes were repeated until the desired number of layers is assembled (4-8).

B) By using a matrix material: I) Alginate: 60 mg sodium alginate was diluted in the DNA template solution from above. The solution was transferred into a disperser. An aerosol was created by the disperser and sprayed into a 2 % CaCl2 solution. After 10 minutes incubation the particle suspension was centrifuges and the supernatant was discarded. The negatively charged alginate particles were coated with polyelectrolyte described under Examples I A but with the addition of 0.5 % CaCl2 in the

coating buffer. The solid alginate core was dissolved by using a calcium free buffer with high sodium concentration in the last step. Optional capsules were treated with homo-bis-functional crosslinking reagent to increase capsule stability.

II) Agarose: 1% low melting agarose (40 degree melting point) was added to the DNA template solution from above and warmed to 45 degree. 100 µl of this solution was added to 2 mL pre-warmed (45 degree) oil. The mixture was shacked to form an emulsion with a droplet diameter of about 50 µm. The emulsion was poured into 10 ml of cold (4 degree) PAH solution (5 mg/mL) and was stirred for some minutes (1 to 20 min). The agarose microbeads were harvested by centrifugation (1200 g for 10 min). The oil phase was removed and the microbeads were washed with cold buffer. The beads were consecutively coated with multiple layers of PSS and PAH.

Example II: Performing of PCR temperature cycling and identification of capsules containing PCR product

Capsule suspensions with 103 to 1012 capsules per ml are diluted with 9 parts PCR buffer containing 2 mM magnesium chloride, 0.2 mM of each dNTP (fluorescent labeled), 1x Taq buffer. PCR cycling is performed in microreaction tubes (50-200 µl per tube) or in microtiter plates (10-100 µl per well) by using conventional cycler.

The PCR cycle set-point temperatures were set as follows: Initial denaturation 90(C for 300 s (Denaturation 92(C for 60 s; Annealing 50(C for 60 s; Elongation 65(C for 60 s) x 15 to 35 cycles.

A flow cytometer with fluorescent detection and a cell sorter are used to identify and to separate capsules containing PCR product. The resulting suspension of capsules containing PCR product was further analysis. For example: the suspension volume was 1 ml and contains 1000 capsules. The suspension was diluted to 750 capsules per milliliter and 10 µl aliquots were dispensed into each well of a 1536 well microtiter plate (Greiner)

resulting in a density of about 0.5 capsules per well (Statistically a very high chance of not more than 1 capsule per well is achieved). After rupturing of capsules (addition of ~100 µl 1 M NaOH, 1 min incubation, addition of the same volume 1 M HCL) PCR products originated from a single strand of template are present in particular wells. Wells containing DNA (=fluorescent labeled PCR product) are easy to identify by fluorescent spectroscopy.

Example III: Applications in nuclear acid analysis and screening

A) Isolating a single target DNA molecule from a complex mixture of DNA molecules:

A complex mixture of DNA molecules (~1010 molecules) was diluted and encapsulated together with the PCR reaction mixture described above, leading to a suspension of about 1020 capsules. Statistically 0.5 to 1 molecule should be present in one capsule. A primer pair designed to amplify a region of the target sequence was added to the template solution prior encapsulation (0.8 µM). The PCR reaction and the isolation of capsules containing PCR product was performed as described in Example II. A positive capsule containing PCR product contains the target DNA molecule (minimum 1 copy) as well. A single positive capsule containing at least one copy of the target DNA can be used for further analysis. The capsule can be destructed and additional target sequences can be identified on the isolated target DNA by PCR.

Claims

1. A nucleic acid amplification process comprising the steps
   (a) providing a nucleic acid amplification reaction mixture,
   (b) encapsulating said reaction mixture by a capsule being permeable for low molecular weight molecules and not permeable for high molecular weight molecules and thereby forming capsules comprising constituents of the reaction mixture, and
   (c) performing at least one amplification or/and polymerization step and thereby amplifying a nucleic acid within one or more of the capsules.

2. The process according to claim 1, further comprising the step
   (d) detecting capsules containing a nucleic acid product.

3. The process according to claim 1 or 2, further comprising the step
   (e) separating capsules containing nucleic acid product from capsules not containing nucleic acid products.

4. The process according to any of the preceding claims, further comprising the step
   (f) analyzing or extracting the nucleic acid product.

5. The process according to claim 1, wherein the constituents of the nucleic acid amplification reaction mixture are incorporated into a matrix material.

6. The process according to claim 5, wherein the matrix material is selected from a porous microparticle, a wax-like substance, an alginate, an agarose, a polysaccharide, a polypeptide, a fatty acid, a

salt of a fatty acid, polyvinyl pyrrolidone (PVP), or the matrix is ice, or a mixture thereof.

7. The process according to any of the preceding claims, wherein the nucleic acid amplification reaction mixture incorporated into a matrix material is encapsulated using a layer-by-layer process, a polymerization process or/and a cavaciation process.

(Delete this claim: The process according to any of claims 1 to 4, wherein the nucleic acid amplification reaction mixture is dissolved in a liquid and encapsulated by liposome formation)

8. The process according to any of claims 5 to 7, wherein the matrix material is dissolved and/or removed from the capsule with solvents, by chemical means or by physical means such as heat.

9. The process according to claims 5 to 7, wherein the matrix material is melted during temperature cycling.

10. The process of any of the preceding claims, wherein capsules are formed having a diameter from 50 nm to 1000 μm, with a preferred diameter of 40 to 60 μm.

11. The process according to any of the preceding claims, wherein constituents and/or reactants of the nucleic acid amplification reaction mixture are transferred into the capsule by a triggered change in capsule wall permeability.

12. The process according to claim 11, wherein the change in capsule wall permeability is triggered by a pH change or an ionic strength change or a change in capsule cross linking.

13. A process according to claim 11 or 12, wherein the permeability of the capsule is controlled to allow selective diffusion of nucleic acid amplification reaction starting materials into the capsules but restrict the out-diffusion of nucleic acid amplification reaction products and/or templates and/or primers.

14. The process according to any of the preceding claims, wherein non-encapsulated nucleic acid amplification reaction mixture compounds are separated from the capsules or destructed by enzymes prior to the amplification step.

15. The process according to any of the preceding claims, wherein one or more components of the nucleic acid amplification reaction mixture and/or of the capsule material carries a label.

16. The process according to claim 15, wherein the label is selected from a fluorophore, a quantum dot, a radioisotope, a dye, a nanoparticle or an NMR active isotope.

17. The process according to claim 15 or 16, wherein the label enables the encoding of individual capsules.

18. The process according to any of the preceding claims, wherein capsules containing nucleic acid products are detected by the physical signature caused by a label or by UV adsorption measurement of DNA, or by dyes staining the DNA, and are separated and isolated from the capsule suspension.

19. The process according to any of the preceding claims for the analysis and/or screening of nucleic acids or artificial derivatives of nucleic acids.

20. The process according to any of the preceding claims for the production of a nucleic acid library.

21. The process of any of the preceding claims employed in a bioassay.

22. The process according to any of the preceding claims, characterized in that the nucleic acid amplification reaction is a polymerase chain reaction (PCR).

23. The process according to claim 22, comprising the steps

(i) providing a PCR reaction mixture consisting of polymerase and/or dNTPs and/or buffer substances and/or enhancers and/or primers and/or templates and/or DNA probes and/or matrix material,

(ii) encapsulating said PCR reaction mixture and thereby forming capsules comprising constituents of the reaction mixture and

(iii) performing at least one amplification step comprising PCR temperature cycling and thereby amplifying a nucleic acid within one or more of the capsules.

24. The process according to any of claims 11-13 or 22-23, wherein the template and/or one or more primers are transferred into the capsule by a triggered change in capsule wall permeability.

25. The process according to any of claims 11-13 or 22-24, wherein the permeability of the capsule is controlled to allow selective diffusion of dNTPs and/or ddNTPs and/or small primers into the capsules but restrict out-diffusion of polymerase, PCR products, templates and large primers during the temperature cycling.

26. The process according to any of claims 22-25, wherein the capsules are dispersed in a PCR buffer.

27. The process according to claim 26, wherein the PCR buffer contains intercalating dyes such as ethidium bromide and/or SYBR Green I.

28. The process according to any of claims 22-27, wherein the temperature cycling of the capsule suspension is carried out in reaction tubes, multititer plates or glass capillaries, using a thermocycler.

29. The process according to any claims, wherein dNTPs and or ddNTPs and/or primers and/or templates and/or DNA probes and/or capsule material carry a label.

30. The process according to any of claims 15-16 or 29, wherein the label enables the encoding of primers and/or templates and/or mixtures thereof.

31. The process according to any of claims 22-30, wherein further enzymes such as reverse transcriptases and/or nucleases and/or ligases are added into the encapsulated PCR reaction mixture.

32. A method of sequencing the nucleic acid content of capsules obtained by any of claims 1-31 comprising the steps:

(i) performing the PCR amplification with an excess of one primer until the primer with the lower concentration is used up.

(ii) adding 4 color fluorescent labeled ddNTPs at an amount of 1:100 to 1:1000 of dNTPs and elongating the excess primer and

(iii) analyzing the nucleic acid capsule content of individual capsules by gel electrophoresis.

33. A method of analyzing the nucleic acid content of capsules obtained by any of claims 1-31 comprising the steps:
    (i) embedding capsules into an agarose or polyacrylamide gel,
    (ii) releasing nucleic acid from the capsule by chemical or physical means.
    (iii) applying an electrical potential to carry out a gel electrophoresis and
    (vi) detecting the band patterns created from individual capsules.

34. Capsule encapsulating a nucleic acid amplification reaction mixture and/or a nucleic acid amplification product obtainable by any of claims 1-31.

35. Capsule according to claim 34, encapsulating a PCR reaction mixture and/or a PCR product.

36. Use of a capsule according to claim 34 or 35 for the analysis and/or screening of nucleic acids or artificial derivatives of nucleic acids, for the production of nucleic acid libraries, nucleic acid sequencing, in bioassays or/and in real-time PCR.

37. Use of the capsules according to claim 34 or 35 as reaction compartments for parallel nucleic acid amplification reactions.

38. Use according to claim 37 as reaction compartment for parallel PCR reactions.

39. A kit for the production of capsules according to any previous claim, and/or the performance of the process of any previous claim and/or the use of capsules according to any previous claim, containing polymerase and/or dNTPs and/or ddNTPs and/or buffer substances and/or enhancers and/or primers and/or templates and/or DNA

probes and/or matrix material and/or capsule material and/or labels and/or plastic ware and/or a user manual.