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(54) Title: METHOD AND DEVICE FOR MIXING TWO STREAMS OF DROPLETS

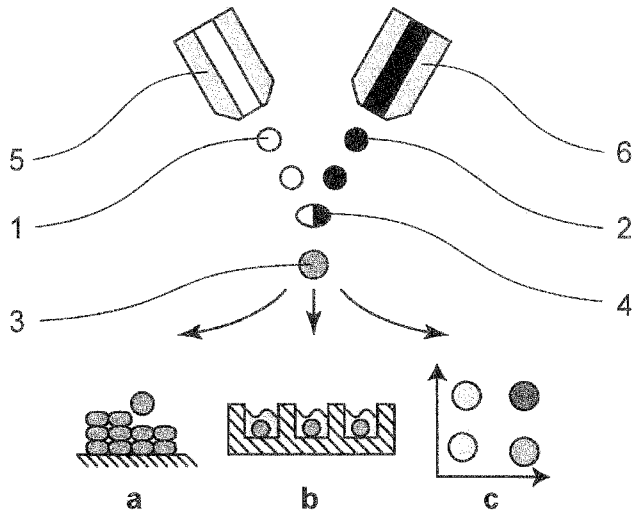

Fig. 1

(57) Abstract: The invention relates to a method for mixing a first stream of droplets (1) with at least one second stream of droplets (2) to obtain a third stream of fused droplets (3), by collision and fusion of at least one droplet of the first stream (1) with at least one droplet of the second stream (2). The first stream of droplets (1) and the at least one second stream of droplets (2) are generated in a synchronized manner. The invention also relates to a device for generating and mixing a first stream of droplets (1) and at least one second stream of droplets (2) to obtain a third stream of fused droplets (3), to a droplet, a plurality of droplets, or to a two-dimensional or three-dimensional scaffold generated by this method. Through the described method and device, it is possible to exploit the soformed fused droplets for several different applications such as 3D patterning (a), high throughput screening (b) and combinatorial studies (c).

Method and device for mixing two streams of droplets

The invention relates to a method for mixing a first stream of droplets with at least one second stream of droplets to obtain a third stream of fused droplets, to a device for generating and mixing a first stream of droplets with at least one second stream of droplets to obtain a third stream of fused droplets, to a droplet, a plurality of droplets or to a two-dimensional or three-dimensional scaffold generated by this method, according to the independent claims.

In recent years, micro-scale mixing has become increasingly important in various fields ranging from biomedical diagnostics and drug development to food and chemical industries. An attractive application, in which the very precise and fast picoliter-range mixing of liquids is required, is the microencapsulation of cells in an ultra-high-throughput fashion. This technique allows the generation of combinatorial cellular microenvironments for the screening and discovery of two dimensional and three dimensional cell culture substrates.

Notably, the application of such substrates has a high potential in stem cell research. Stem cells are drawing increasing attraction of the scientific community for having the potential to expand and generate many types of tissue specific cells. However, such potential is strongly related to the microenvironment in which they reside in-vivo, the so-called niche. To recapitulate such a complex network of cues, stem cell biologists need greater control over cell culture conditions. Furthermore, stem cells usually show high heterogeneity when kept in culture. In order to systematically study such populations, single cell manipulation becomes a crucial requirement.

On the other hand, micro-scale mixing is an important aspect to be addressed in the fabrication of three dimensional artificial tissues. In this context, ink jet printers have been increasingly exploited to handle micro-volumes of biologically relevant solutions. Importantly, high cell compatibility of such tools has been achieved. Cells have been printed without negatively affecting their viability within a multitude of biomaterials. A typical droplet size of less than 100 µm is appropriate to contain a single mammalian cell (smaller than 25 µm). This parameter can be controlled through modification of the dispensing parameters.

De Gans and Schubert have provided a comprehensive review on commercially available instrumentation for ink jet printing of polymer micro-arrays (Macromol. Rapid Commun. 2003, 24, 659-666). They explain that even with nowadays commercially available ink jet equipment, a number of problems remains unsolved, most notably the problem of how to conduct the mixing of components when creating micro-arrays of polymer blends. There are three main choices to address this issue: mixing before printing, mixing during printing or mixing on the substrate. In order to effect mixing during printing, the collision of two different droplets in air is described. However, according to de Gans and Schubert, this approach is far too complicated to be applicable in a routine practice.

The patent application US 2003/0175410 A1 describes methods and apparatuses for selectively depositing so-called bio ink solutions in order to build up a three-dimensional biomimetic scaffold. In particular, a method is provided for preparing such scaffolds by co-depositing two or more of bio ink solutions. These structures are prepared using a solid free form fabrication system (i.e. a 3D printer) comprising an apparatus employ-

ing one or more focused micro dispensing devices, which permits the co-depositing of bio inks in controllable manner. During operation, a first micro dispensing device and a second micro dispensing device may selectively dispense a focused volume of a first bio ink and of a second bio ink at a plurality of dispensing locations on a surface. Hence, the bio inks are mixed up on deposition on the surface. This technology can be used to cause solidification or gelation of the bio ink in order to produce the desired three-dimensional structure of a biomimetic scaffold.

However, the described system has the drawback that the mixing of the bio inks occurs in a rather uncontrolled manner. Consequently, the solidification or gelation of the bio inks, for instance the formation of a hydrogel by chemical or enzymatic reactions, proceeds relatively slow. This attenuates the resolution of such a device. Furthermore, micro encapsulation of living cells cannot be achieved and patterning of the material is impossible.

It is a problem underlying the present invention to overcome these drawbacks in the state of the art.

In particular, it is a problem underlying the present invention to provide methods and means to effect reliable and precise micro-scale mixing of multiple components, even in routine applications. The mixing should be compatible with physiological systems, in particular with living cells. It should be cost-efficient and safe. Furthermore, a high degree of automation is desirable and the mixing technology should allow for high-throughput applications. It should also allow for the mixing of a wide variety of different liquids in nanoliter quantities. Moreover, it is a problem underlying the present invention to

provide corresponding micro-scale droplets with improved properties and enhanced two-dimensional or three-dimensional scaffolds.

These problems are solved by a method according to claim 1, a device according to claim 16 and products according to claims 19 and 20.

The present invention refers to a method for mixing a first stream of droplets with at least one second stream of droplets to obtain a third stream of fused droplets by collision and fusion of at least one droplet of the first stream with at least one droplet of the second stream. The method is characterized in that the first stream of droplets and the least one second stream of droplets are generated in a synchronized manner.

By directly colliding and fusing at least two droplets, efficient micro-scale mixing is achieved. To this end, the synchronized generation of the droplets is of critical importance in order to achieve controlled collision and fusion. In the present context, the term "synchronized generation" refers to a generation of two or more streams of droplets in a manner that substantially all droplets of the first stream undergo collision and fusion with a droplet of a second stream, and vice versa. The term "substantially all" refers to a ratio of at least 85%, preferably 90%, even more preferably 95% or 99%. As the droplets are generated and mixed streamwise, the method is suitable for high-throughput applications.

At least one of the streams of droplets can be generated by a piezoelectric or thermal ink jet dispenser. The ink jet technology is a well-established method for the generation of droplets in a picoliter volume range and allows for the formation of

droplets in a very high frequency and a narrow size distribution.

The collision and the fusion of at least one droplet of the first stream with at least one droplet from a second stream can occur in a flying phase. In a flying phase collision and fusion, the incoming flying trajectories of at least two droplets combine to a final trajectory that is a combination of the original ones. This in-flight-mixing allows the union of two droplets without any interference of a support-vessel or - surface.

Upon collision and fusion, a reaction, in particular a cross-linking-reaction, can occur between a first component contained in the first stream of droplets and a second component contained in a second stream of droplets. Accordingly, the mixing-technique provides an attractive approach for conducting various chemical or biological reactions on a picoliter-scale.

A crosslinking-reaction can occur to form a stream of hydrogel droplets. This allows mixing two precursor-solutions of a hydrogel in order to form a hydrogel droplet under well-defined conditions (e.g. volumes and mixing-time). In this context, a hydrogel is a highly swollen network of cross-linked polymer chains with the swelling agent water. Hydrogels can contain over 90% of water. They possess a degree of flexibility which is very similar to natural tissue. This allows very broad application of hydrogels as scaffolds in tissue engineering.

The hydrogel droplets can be collected in a liquid bath or deposited on a substrate. This underlines the high flexibility of this method.

The hydrogel droplets can be deposited on a substrate to generate a two-dimensional or three-dimensional scaffold. Such a scaffold can have tissue-like characteristics. The method is therefore amenable for tissue engineering.

The first component can contain a naturally derived macromolecule, preferably an alginate, and a second component can be a solution of a crosslinking ion, preferably a metal ion, even more preferably a calcium ion. The crosslinking-reaction of alginates with calcium ions proceeds with very short reaction times. The formed hydrogels exhibit high biocompatibility and allow for various applications, in particular in tissue engineering.

On the other hand, the first component can contain a synthetic macromolecule, in particular a PEG-based polymer, preferably modified with oligopeptides, and a second component can be a solution of a crosslinking agent, preferably an enzyme, even more preferably a transglutaminase.

Such a PEG-hydrogel provides an attractive alternative to an alginate-based hydrogel. Even though the reaction times for crosslinking are usually longer, such hydrogels show an increased stability. Furthermore, these hydrogels can be readily tethered with biologically active signals.

The first component can also contain a reactive synthetic macromolecule, preferably a PEG-based polymer, comprising maleimidie or vinylsulfonate functional groups, in particular at terminal positions. In such an application, a second component can contain a counter-reactive hydrogel precursor, preferably a synthetic macromolecule, in particular a PEG-based polymer, comprising thiol groups, in particular at terminal positions. How-

ever, a second component can also contain a cysteine containing peptide instead, in particular an oligopeptide or a polypeptide. These hydrogel systems have beneficial properties. Their cross-linking reactions proceed with sufficiently short reaction times for the formation of three-dimensional structures, in particular in a layer-by-layer fashion. Furthermore, they form peptidic substrates for proteases, in particular for metalloproteases.

Importantly, the crosslinking kinetics of such systems can be increased by blending PEG-based hydrogels with alginate, which, if desired, can be readily removed after PEG crosslinking by lyase treatment. This "hybrid hydrogel" strategy is advantageous because both hydrogel systems share calcium (Ca2+) as the common entity enabling crosslinking. That is, the alginate component can be rapidly cross-linked to form a framework within which the PEG component may cross- link via a reaction which proceeds more slowly. The alginate framework ensures that the PEG hydrogel retains a three-dimensional structure while the slower PEG cross-linking reaction proceeds. The alginate may then be removed to leave only the PEG hydrogel.

It should be noted that the above-mentioned hybrid hydrogel network concept can be implemented with other slowly crosslinkable hydrogel systems. Accordingly, the second component can further comprise a macromolecule, preferably a naturally-derived macromolecule, in particular fibrinogen or hyaluronic acid. As such, a wide range of hydrogel systems can be tailored to be useful for in situ droplet mixing.

At least one of the first stream of droplets and the second stream of droplets can contain a, preferably mammalian, living cell that is encapsulated into the hydrogel droplet. The in-vivo encapsulation of cells is particularly attractive in stem cell

research to provide a well-defined microenvironment. Furthermore, the encapsulated cells can also be deposited in a two-dimensional or three-dimensional scaffold to generate a tissue like artificial living structure.

On the other hand, the first stream of droplets can contain at least one, preferably mammalian, living cell of a first type and a second stream of droplets can contain at least one, preferably mammalian, living cell, in particular of a second type, the cells are then co-encapsulated into a hydrogel droplet. The co-encapsulation of living cells into the same hydrogel droplet is an attractive approach for studying cell-cell interactions (whether of cells of the same type, or cells of different types).

Another aspect of the present invention refers to a device for generating and mixing a first stream of droplets with at least one second stream of droplets to obtain a third stream of fused droplets. The mixing preferably proceeds by an above described method. The device comprises

- a first dispenser for generating the first stream of droplets,
- at least one second dispenser for generating at least one second stream of droplets,
- holding means for holding the first and at least one second dispenser.

The first and/or at least one second dispenser preferably is/are a piezoelectric or thermal ink jet dispenser(s).

The device is characterized in that it further comprises synchronizing means for synchronizing the operation of the first

and at least one second dispenser to effect collision and fusion of at least one droplet of the first stream with at least one droplet of the second stream to obtain the third stream.

Such a configuration allows for the efficient and reliable mixing of droplets. In particular, collision and fusion of substantially all droplets of the first stream with a droplet of a second stream, and vice versa, can be achieved.

Synchronization of the operation of the first and at least one second dispenser can be achieved by a reiterative process comprising the steps of:

- mechanically adjusting the nozzle positions inside the housings
- establishing continuous flows from the nozzles
- visually assessing crossing of the flows
- repeating the procedure until proper crossing is achieved
- once proper crossing is achieved, setting the ejection speed at each dispensing unit to the same value, by acting on dispensing parameters, such as voltage and pulse length.

The holding means can be suitable to adjust the relative position and orientation of the first dispenser and the at least one second dispenser. This way, proper mixing can be attained by tuning the geometry of droplet collision. The angle of impact can be adapted to the energy of the incoming droplets, in order to promote fusion and to avoid re-separation of the droplets due to their inertial energy. The angle between the alignment axis of the first dispenser and the alignment axis of the second dispenser can be adjustable in a range from 30° to 90°. Moreover, the possibility to tune the droplet-generation by acting on voltage, pulse-length and frequency allows properly synchroniz-

ing the incoming jets of droplets and modifying droplet characteristics (volume and speed) in order to obtain a precise mixing ratio upon the impact to finally control the mixing parameters.

The invention also relates to a droplet or a plurality of droplets generated by an above-described method. Such droplets have superior characteristics, as they exhibit a mixing-homogeneity that cannot be achieved by conventional methods.

Furthermore, the invention also relates to a two-dimensional or three-dimensional scaffold generated by an above-described method. Such scaffolds are valuable structures for tissue-engineering.

Further advantages and features of the invention are apparent from the following description of several embodiments and in the figures.

It is shown in

Figure 1: Schematic representation of the in-flight-mixing technique with several application examples;

Figure 2: Formation of hydrogel droplets by a cross-linking reaction and collection of the droplets in a liquid bath;

Figures 3 to 5: Series of microscopic images representing a droplet fusion and mixing process according to the present invention.

Figure 1 provides a schematic overview of an in-flight-mixing technique for two streams of droplets according to the present invention. The first stream of droplets 1 is generated by the first dispensing unit 5 and the second stream of droplets (2) is generated by the second dispensing unit 6. The streams of droplets are collided to form a fused droplet 4 in which a crosslinking reaction occurs to form a stream of hydrogel droplets 3. The hydrogel droplets 3 can be used for several applications, such as 3D-patterning a, high throughput screening b or combinatorial studies c. In high throughput screening, it is possible by this technique to create a multitude of replicates of the same microenvironment and screen at series of different extrinsic factors. Combinatorial studies represent an upgrade of the high throughput screening application, in which several variants of compositions are generated and screened according to specific requirements. Finally, 3D-patterning can be achieved by controlling the deposition of the in-situ formed bio ink according to precise blueprints. 3D-patterning can also be combined with combinatorial microenvironment formulation in order to create 3D-scaffolds with heterogeneous composition and function.

Figure 2 shows a further embodiment of the present invention in which a living cell 9 is encapsulated into a hydrogel droplet 3. To this end, a first stream of droplets 1 containing single living cells is collided with a second stream of droplets 2 to generate a fused droplet 4 in which a crosslinking reaction of the hydrogel precursors occurs to form a stream of hydrogel droplets 3. These hydrogel droplets are collected in a liquid bath 7. The enlarged representation of a hydrogel droplet 3' shows that it contains several niches 8. One of those niches is occupied with the living cell 9.

Figures 3 to 5 show a series of microscopic photographs representing the droplet collision and fusion process according to the present invention. In figure 3, the impact of a first droplet of the first stream 1 with a second droplet of a second stream 2 is shown. Figure 4 shows the first phase of the droplet fusion process. It can be seen that at this stage, the fused droplet 4 does not have a spherical shape yet. Figure 5 shows the second phase of the droplet fusion process in which the fused droplet 4' already has a spherical shape. The mixing of the droplets immediately commences upon the impact (Fig 3) and finishes at the late stage of phase two (Fig. 5).

Claims

1. A method for mixing a first stream of droplets (1) with at least one second stream of droplets (2) to obtain a third stream of fused droplets (3) by collision and fusion of at least one droplet of the first stream (1) with at least one droplet of the second stream (2), characterized in that, the first stream of droplets (1) and the at least one second stream of droplets (2) are generated in a synchronized manner.

2. The method according to claim 1, characterized in that, at least one of the streams of droplets is generated by a piezoelectric or thermal ink jet dispenser.

3. The method according to claims 1 or 2, characterized in that, the collision and the fusion of the at least one droplet of the first stream (1) with at least one droplet of a second stream (2) occurs in a flying phase.

4. The method according to one of claims 1 to 3, characterized in that, upon collision and fusion, a reaction, in particular a crosslinking-reaction, occurs between a first component contained in the first stream of droplets (1) and a second component contained in a second stream of droplets (2).

5. The method according to claim 4, characterized in that, the crosslinking-reaction occurs to form a stream of hydrogel droplets.

6. The method according to claim 5, characterized in that, the hydrogel droplets are collected in a liquid bath (7) or deposited on a substrate.

7. The method according to claim 5, characterized in that, the hydrogel droplets are deposited on a substrate to generate a two-dimensional or three-dimensional scaffold.

8. The method according to one of claims 5 to 7, characterized in that, the first component contains a naturally derived macromolecule, preferably an alginate, and a second component is a solution of a crosslinking ion, preferably a metal ion, even more preferably a calcium ion.

9. The method according to one of claims 5 to 8, characterized in that, the first component contains a synthetic macromolecule, in particular a PEG-based polymer, preferably modified with oligopeptides, and a second component is a solution of a crosslinking agent, preferably an enzyme, even more preferably a transglutaminase.

10. The method according to one of claims 5 to 9, characterized in that, the first component contains a reactive synthetic macromolecule, preferably a PEG-based polymer, comprising maleimidie or vinylsulfonate functional groups, in particular at terminal positions.

11. The method according to claim 10, characterized in that, a second component contains a counter-reactive hydrogel precursor, preferably a synthetic macromolecule, in particular a PEG-based polymer, comprising thiol groups, in particular at terminal positions.

12. The method according to claims 10 or 11, characterized in that, a second component contains a cysteine containing peptide, in particular an oligopeptide or a polypeptide.

13. The method according to claim 8, characterized in that, the second component further comprises a macromolecule, preferably a naturally-derived macromolecule, in particular fibrinogen or hyaluronic acid.

14. The method according to one of claims 5 to 13, characterized in that, at least one of the first stream of droplets (1) and the second stream of droplets (2) contains a, preferably mammalian, living cell (9) that is encapsulated into the hydrogel droplet.

15. The method according to one of claims 5 to 14, characterized in that, the first stream of droplets (1) contains at least one, preferably mammalian, living cell (9) of a first type and the second stream of droplets (2) contains at least one, preferably mammalian, living cell, in particular of a second type, and that the cells are co-encapsulated into the hydrogel droplet.

16. A device for generating and mixing a first stream of droplets (1) with at least one second stream of droplets (2) to obtain a third stream of fused droplets (3), wherein the mixing preferably proceeds by a method according to any one of claims 1 to 15, comprising:

- a first dispenser (5) for generating the first stream of droplets (1)
- at least one a second dispenser (6) for generating at least one second stream of droplets (2),
- holding means for holding the first and at least one second dispenser

wherein the first (5) and/or the at least one second dispenser (6) preferably is/are (a) piezoelectric or thermal ink jet dispenser(s), characterized in that, the device fur-

ther comprises synchronizing means for synchronizing the operation of the first (5) and the at least one second dispenser (6), to effect collision and fusion of at least one droplet of the first stream (1) with at least one droplet of the second stream (2) to obtain the third stream (3).

17. The device according to claim 16, characterized in that, the holding means are suitable to adjust the relative position and/or orientation of the first dispenser (5) and the at least one a second dispenser (6).

18. The device according to claim 17, characterized in that, the angle between the alignment axis of the first dispenser (5) and the alignment axis of the second dispenser (6) are adjustable in a range from 30° to 90°.

19. A droplet or a plurality of droplets generated by a method according to any one of claims 4 to 15.

20. A two-dimensional or three-dimensional scaffold generated by a method according to any one of claims 4 to 15.

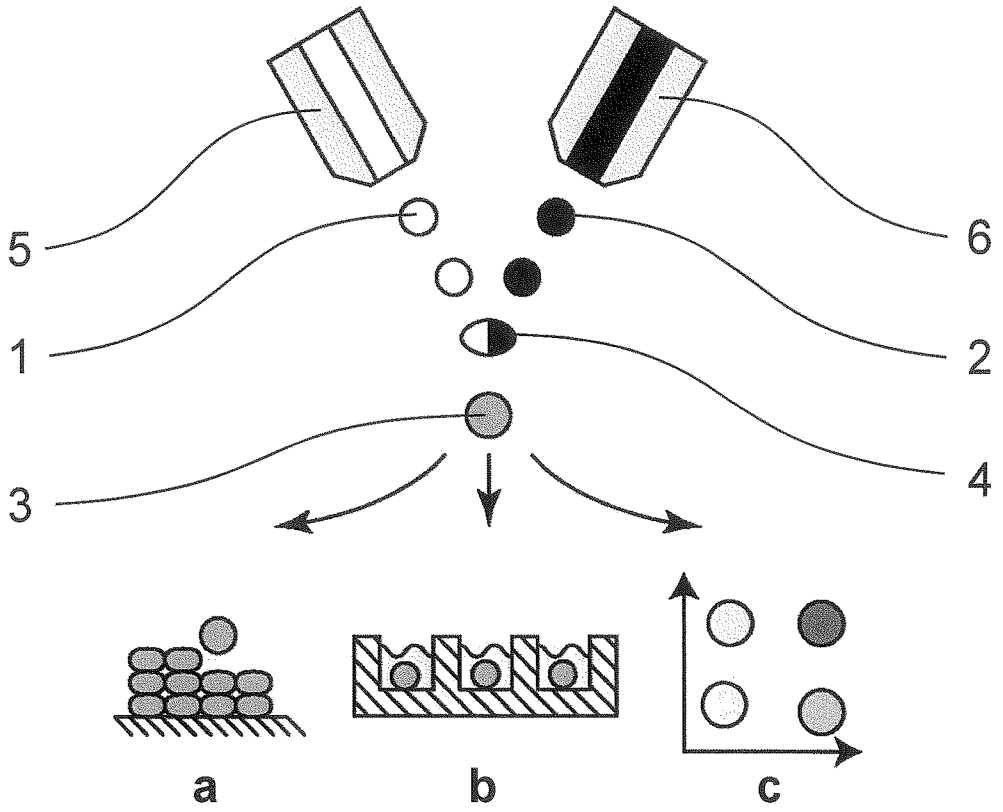

Fig. 1

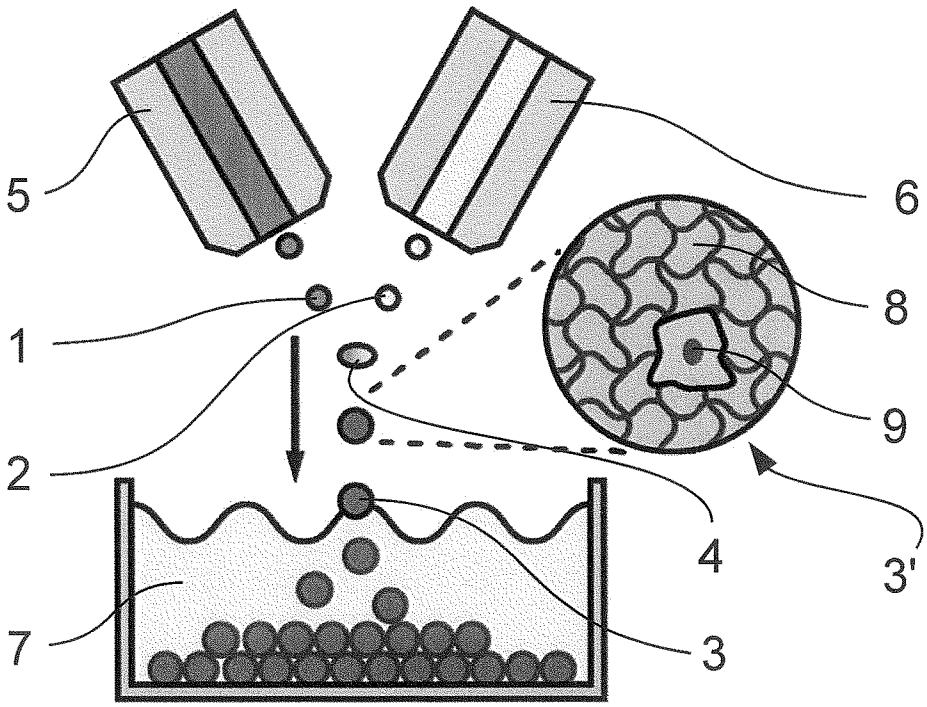

Fig. 2

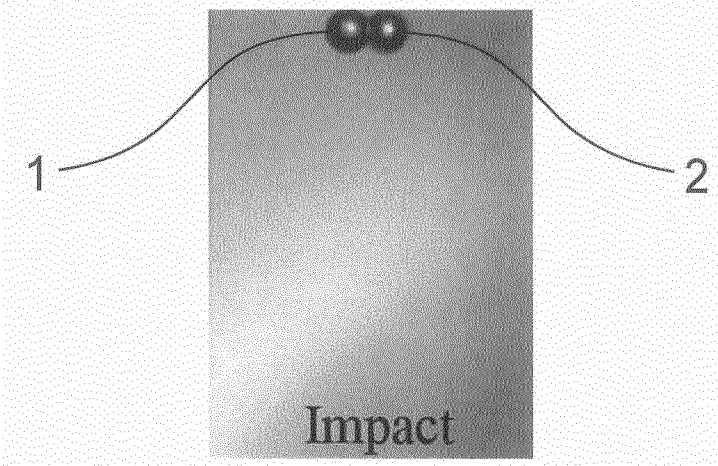

Fig. 3

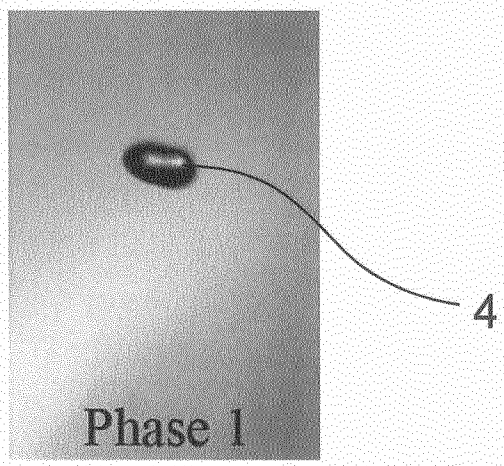

Fig. 4

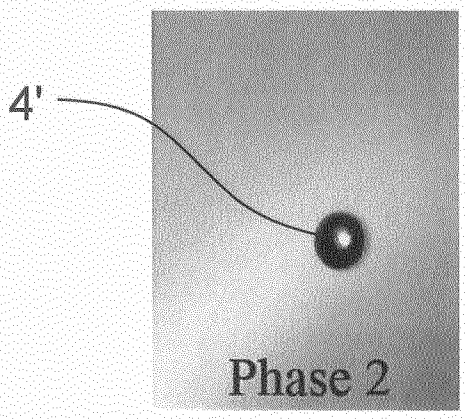

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/078881

A. CLASSIFICATION OF SUBJECT MATTER
INV. B01F5/02 B01F5/00 A61L27/38 A61L27/60 C12N5/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
B01F A61L C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 2 444 147 A1 (SONY CORP [JP]) 25 April 2012 (2012-04-25) paragraph [0001] paragraph [0006] - paragraph [0007] paragraph [0010] - paragraph [0012] paragraph [0016] - paragraph [0021] paragraph [0107] - paragraph [0108] figures 1,2,3 ----- -/--	1-4, 16-19 5-15,20

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 2015	15/09/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Real Cabrera, Rafael

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/078881

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/056860 A1 (POSTECH ACAD IND FOUND [KR]; KWON TAI-HUN [KR]; PARK SUNG-JEA [KR]) 15 May 2008 (2008-05-15)	1-3, 16-18
A	paragraph [0001] paragraph [0006] paragraph [0014] - paragraph [0015] paragraph [0026] - paragraph [0027] paragraph [0032] - paragraph [0033] paragraph [0053] - paragraph [0062] paragraph [0095] - paragraph [0108] figures 3,10-15	4-15,19, 20

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A	column 1, line 6 - line 16 column 1, line 39 - line 50 column 3, line 55 - column 4, line 22 figures 1,2	4-15,19, 20

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Y	paragraph [0001] paragraph [0006] - paragraph [0007] paragraph [0027] - paragraph [0028] figure 2	4-15,19, 20

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A	paragraph [0002] paragraph [0008] - paragraph [0009] paragraph [0081] paragraph [0085] - paragraph [0087] paragraph [0218] - paragraph [0220]	1-3, 16-18

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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