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Co-precipitation of metal carbonate templated protein microparticles – using the KNAUER IJM NanoScaler

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SUMMARY

The KNAUER IJM NanoScaler can be used to precipitate protein metal carbonate hybrid particles by mixing two inorganic salt solutions ($MnCl_2$ and Na_2CO_3) in which the protein is also dissolved. Adding suitable additives allows the production of such hybrid particles in µm range mainly as single particles with only a small number of aggregates.

INTRODUCTION

The Co-precipitation-Crosslinking-Dissolution (CCD) technique allows a few-step-fabrication of particles composed of different biopolymers and bioactive agents under mild conditions without usage of any organic solvents [1, 2]. When mixing two or more inorganic salt solutions (e.g. $MnCl_2$ and Na_2CO_3) in which the biopolymers are also dissolved, water-insoluble template particles/metal carbonate particles are formed and the biopolymers are encapsulated in the particles during precipitation (co-precipitated hybrid particles) [1]. These hybrid particles are treated with a suitable crosslinker so that the biopolymers encapsulated in the template are crosslinked. After washing, the particles are further treated with a solution that can dissolve the inorganic template (e.g. EDTA, hydrochloric acid). After purification, pure biopolymer particles are obtained without the inorganic salt originally used [1]. Currently, the CCD technique is being implemented to develop hemoglobin and albumin particles that can act as oxygen- or drug-carriers [2–7].

The co-precipitation step is the key process of the CCD technique and largely determines the size and morphology of the biopolymer particles and their yield. The co-precipitation step can be carried out manually in the laboratory by pouring the two inorganic salt solutions together while stirring. However, this method is not standardized. For rigorous product development, it is imperative to implement a standardized process for co-precipitation that complies with "good manufacturing practice" (GMP) and that is scalable and automatable. Here, we investigate whether the traditional co-precipitation step of the CCD method, in which bulk solutions are manually mixed in a beaker, can be replaced by automatized mixing with the Impingement Jets Mixing (IJM) technology of the NanoScaler. The size of the precipitated protein particles should be adjustable in the range of 0.1 to 10 µm.

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SAMPLE PREPARATION

0.25 M $MnCl_2$ containing 10 mg/ml hemoglobin and 0.25 M Na_2CO_3 were mixed using KNAUER IJM #5. 0.5 mg/ml human serum albumin (HSA) solution was used to dilute/block the precipitation. Different flow rates and additives were tested **(Tab. 1)**. Samples were analyzed by dynamic light scattering (DLS) and confocal laser scanning microscopy (CLSM).
After each experiment, to avoid clogging by remaining solution in the system, the NanoScaler was immediately washed with distilled water, followed by flushing with 0.5 M EDTA pH 7.4, distilled water again and 30 % ethanol.

Tab. 1 Flow rates, additives used, and final concentration of additive

Flow rate (ml/min)		
$MnCl_2$-Hb/Na_2CO_3/HSA	Additive in Na_2CO_3 solution	Final concentration of additive in precipitate before dilution with HSA
1/1/3	–	
2/2/6	–	
2/2/6	Hyaluronic acid (HA)	0.1 %
2/2/6	Hydroxyethyl starch (HES)	2 %
2/2/6	Oxidized dextran 70 (ODx)	2 %
2/2/6	Dextran 70 (Dx)	2 %
2/2/6	PEG 10kD	2 %
1/1/3	Dextran 70	2 %
3/3/9	Dextran 70	2 %
5/5/15	Dextran 70	2 %
10/10/30	Dextran 70	2 %
12/12/30	Dextran 70	2 %

RESULTS

With a flow rate of 2 ml/min for both $MnCl_2$-Hb and Na_2CO_3 solutions, followed by dilution with 6 ml/min HSA solution, Hb-$MnCO_3$ hybrid particles with aggregation are produced **(Fig. 1)**.

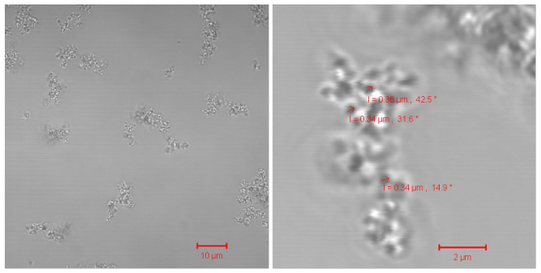


Fig. 1 CLSM images of Hb-$MnCO_3$ hybrid particles produced with KNAUER IJM #5. Flow rate of $MnCl_2$-Hb/Na_2CO_3: 2 ml/min, HSA: 6 ml/min. Particles are aggregating.

Adding 0.2–4 % biopolymer (e. g. hyaluronic acid, PEG, dextran) into the Na_2CO_3 solution caused an increase in size of single particles up to 2.5 µm, but the aggregation of hybrid particles decreased. A 2 % dextran solution added during the precipitation with an increased flow rate (10 ml/min for both $MnCl_2$-Hb and Na_2CO_3 solutions, 30 ml/min for HSA solution), led to formation of spherical hybrid particles with size range of 1–2 µm and with reduced aggregation, as observed under CLSM **(Fig. 2)**.

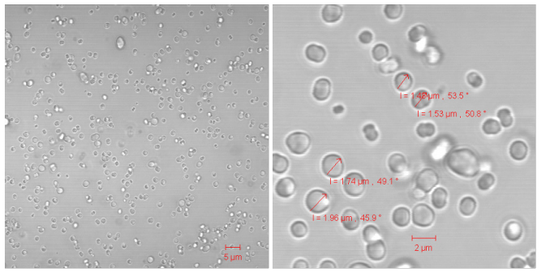


Fig. 2 CLSM images of Hb-$MnCO_3$ hybrid particles produced with KNAUER IJM # 5. Flow rate of $MnCl_2$-Hb/dextran-Na_2CO_3: 10 ml/min, HSA: 30 ml/min. Dextran final concentration before dilution with HSA: 2 %. Particle sizes range between 1–2 µm.

DLS measurements of hybrid particles with dextran as an additive indicate a narrow size distribution by number **(Fig. 3)**.

Size distribution of Hb-$MnCO_3$ hybrid particles produced with IJM #5

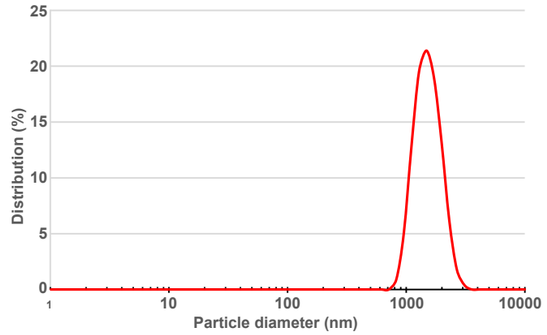


Fig. 3 Flow rate of $MnCl_2$-Hb/dextran-Na_2CO_3: 10 ml/min, HSA: 30 ml/min. Dextran final concentration before dilution with HSA: 2 %

CONCLUSION

It is possible to precipitate protein-metal carbonate hybrid particles with low aggregation using a KNAUER IJM NanoScaler if suitable additives, such as dextran, are used. Currently, hybrid particles with low aggregation in the µm range can be produced. Other polymers as additives or block solution and IJM mixing chambers of varying size, are currently being tested.

MATERIAL AND METHODS

Substances

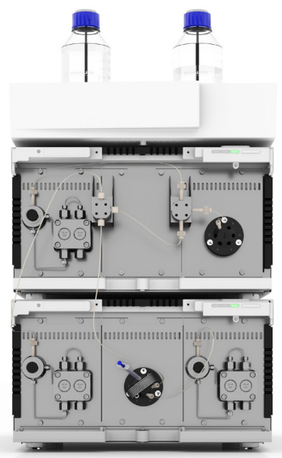
Bovine hemoglobin (Actoheme®) was provided by Biophyll GmbH, Dietersburg, Germany. Human serum albumin (Plasbumin®20) was purchased from Grifols Deutschland GmbH, Frankfurt am Main, Germany. Manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), sodiumcarbonate (Na_2CO_3), ethylene-diaminetetraacetic acid (EDTA), hyaluronic acid sodium salt, PEG 10kD, sodium hydroxide and sodium (meta) periodate were were purchased from Sigma-Aldrich, Darmstadt, Germany. Ethanol was purchased from Carl Roth GmbH, Karlsruhe, Germany. Ampuwa® (aqua ad injectable) was purchased from Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany. Dextran (from Leuconostoc mesenteroides, M.W. 70kDa) was purchased from AppliChem GmbH (Darmstadt, Germany). Hydroxyethyl starch (HES) 130/0,42 was provided by Serumwerk Bernburg AG, Bernburg, Germany. Oxidized dextran 70KD was synthesized as described previously[4].

Method

Confocal laser scanning microscopy (CLSM)
CLSM images were taken with a confocal microscope LSM 510 Meta (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a 100 x oil-immersion objective (numerical aperture 1.3) applying excitation wavelength of 488 nm and a long pass emission filter 505 nm. Magnification: 1000 x.

Dynamic light scattering (DLS)
The hydrodynamic size of hybrid particles was determined using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). Each sample was diluted 1:200 using Ampuwa®. All the measurements were carried out in triplicate.

Instrument	Description	Article No.
NanoScaler		A48040C



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