Optimization of Natural Polyphenol Microcapsule Generation *via* Sonochemical Process for Pharmaceutical Applications

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Abstract

The ultrasonication or sonochemical process is based on the application of ultrasound energy to agitate particles in a liquid medium. It has proven extremely effective in producing core- shell structures with high encapsulation potential. However, for pharmaceuticals applications, the high quantities of microcapsules and the awareness of their statistical mean characteristics are mandatory. Therefore, the optimization of natural polyphenol microcapsule generation *via* sonochemical process is relevant.
For this purpose, representative natural polyphenols were selected and the pH value, natural polyphenol concentration, water/oil phase ratio, ultrasonication power as well as ultrasonication time were varied.
The results of this screening led to the assumption that pH is eventually very crucial in natural polyphenol microcapsules generation via sonochemical process. Overall, a 1:1 mixture of an aqueous natural polyphenol solution (0.5% w/v, normalised to approximately 0.05 mmol/mL of the total phenolic OH-group) at pH~12 and the oily phase, first magnetically stirred for five minutes and subsequently treated by ultra-sonication at 160 W (40% amplitude) for ten minutes, was found to be the most efficient set-up for the generation of natural polyphenol microcapsules.
With these optimized conditions, it was possible to process and isolate microcapsules from lignins like softwood kraft lignin and softwood lignosofonate and tannins like *Acacia mearnsii* bark extract, *Acacia mearnsii* sulfited tannin and *Acacia dealbata* (winter mimosa) tannin and, more importantly also from epigallocatechin gallate of *Camellia sinensis* (green tea) and tannic acid of *Caesalpinia spinosa* that from previous study it was possible to generate only nanoemulsions via the ultrasound assisted method.

Key Words : Natural Polyphenols, Lignins, Tannins, Microcapsules, Microencapsulation and Ultrasonication.

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(Optimization de la Génération de Microcapsules de Polyphénols Naturels par Processus Sonochimique pour des Applications Pharmaceutiques)

Résumé

Le processus d'ultrasonication ou sonochimique est basé sur l'application des ultrasons pour agitation des particules en milieu liquide. Ce processus s'est avéré efficace dans la production des microcapsules à fort potentiel d'encapsulation. Par ailleurs, pour l'ingénierie pharmaceutique où les grandes quantités de microcapsules et la connaissance de leurs caractéristiques morphologiques sont très importantes, l'étude d'optimisation de la production de microcapsules de polyphénols naturels par ce processus est bien pertinente.

Pour ce faire, des polyphénols naturels représentatifs ont été sélectionnés et la valeur du pH, la concentration en polyphénols naturels, le rapport de phase eau/huile, la puissance d'ultrasonication ainsi que le temps d'agitation avec les ultrasons ont été variés.

Les résultats de ce criblage conduit à l'hypothèse selon laquelle le pH est éventuellement très crucial dans la génération de microcapsules de polyphénols naturels par le processus sonochimique. Dans l'ensemble, un mélange 1:1 d'une solution aqueuse de polyphénols naturels (0,5% p/v, normalisée à environ 0,05 mmol/mL du groupe OH phénolique total) à pH ~ 12 et la phase huileuse, d'abord agitée magnétiquement pendant cinq minutes puis traitée par ultra sonication à 160 W (amplitude de 40%) pendant dix minutes, s'est avérée être la configuration la plus efficace pour la génération de microcapsules de polyphénols naturels.

Grâce à ces conditions optimisées, il a été possible de traiter et d'isoler des microcapsules à partir de lignines telles que la lignine kraft de résineux et le lignosofonate de résineux et de tanins tels que l'extrait d'écorce d'*Acacia mearnsii*, le tanin sulfité d'*Acacia mearnsii* et le tanin de *Acacia dealbata* (mimosa d'hiver) et, plus important encore, le gallate d'épigallocatéchine de *Camellia sinensis* (thé vert) et l'acide tanique de *Caesalpinia spinosa* qui, d'après des études précédentes, il n'a été possible de générer que des nanoémulsions via la méthode assistée par ultrasons.

Mots clés : Polyphénols naturels, Lignines, Tanins, Microcapsules, Microencapsulations et Ultrasons.

Introduction

Historically, the microencapsulation technique was first proposed by Chang in the 1960s, in order to encapsulate bioactive materials such as enzymes, proteins and cells in semi-permeable microcapsules (MCs) (1). A microcapsule can be defined as a small sphere with a uniform wall around it. The material inside the microcapsule is referred to as the core or internal phase whereas the wall is sometimes called a shell, coating, or membrane (2).

The idea of using the microencapsulation technique in controlled release process lead to the development of some strategies for the administration of drugs with poor solubility and bioavailability issues (3–5). These aspects support the requirement of reservoir type polymeric micro- or nanosized devices. For instance, the protection of orally administered drugs from the attack of acids in the stomach before they can be absorbed in the intestine is exerted by gelatine microcapsules, which are insoluble at the low pH values found in the stomach allowing the desired release later in the intestinal tract (6).

For the generation of MCs, the selection of an appropriate encapsulation process is mandatory.

The ultrasonication or sonochemical process is one of the most common and well-known micro- and nano-encapsulation, techniques (7), which is referred to the development of loaded microcapsules by means of sonication, *i.e.*, a technique based on the application of ultrasound energy to agitate particles in a liquid medium. This process has proven extremely effective in producing core shell structures with high encapsulation potential (8).

To be qualified for their potential pharmaceutical applications, the natural polyphenol microcapsules (NP-MCs) have to possess some particular characteristics, including a suitable size profile and large quantities. Furthermore, with respect to an use in pharmaceutical applications which normally come with significantly higher demands concerning safety and biocompatibility, tannins are much more useful. Some investigations in this respect have been reported (9–13).

The present work was aimed to increase the quantities of the NP-MCs generation as well as to elucidate the statistical characteristics of the NP-MCs namely the mean diameter, the minimum and maximum diameters, the NP-MCs number, the polydispersity index (PDI) of the NP-MCs diameter. This objective should be achieved by selecting representative NPs and systematically varying the value of pH, NP concentration, water/oil phase ratio, ultrasonication power and time of a previously reported procedure (14), in which the NPs were dissolved in distilled H_2O and an oil in water emulsion and submitted to ultrasonication for the generation of NP-MCs.

I. Materials and Methods

➢ Chemicals and reagents

Chemicals and solvents were purchased from Sigma-Aldrich or Carlo Erba in appropriate grades and were used without further purification if not

stated otherwise. Softwood lignosulfonate (SLS) was obtained from Borregard; softwood kraft lignin (SKL) from the French Institute of Technology 'Forêt Cellulose Bois-construction Ameublement' (FCBA). Purified epigallocatechin gallate (EGCG) form *Camellia sinensis* was used as purchased from a local pharmacy. The *Acacia dealbata* bark extract (*Mim*T) was obtained from Figli di Guido Lapi. The *Acacia mearnsii* bark extract (*Am*T), sulfited *Acacia mearnsii* tannin (*Am*ST), TA, tannic acid and CAT, catechin were purchased from Silvachimica s.r.l.

➢ General Procedures

According to a previously reported procedure (9,14,15), the NPs were dissolved in distilled H_2O and an oil in water emulsion was generated and submitted to ultrasonication. Therefore, for generating higher quantities of capsules, this procedure has been re-visited and optimized by systematically varying the value of pH, NP concentration, water/oil phase ratio, ultrasonication power and time.

Furthermore, based on the primary results obtained in the generation of SKL-MCs as well as *Am*T-MCs, and considering the particular relevance of the beneficial properties of tannins with respect to a function as shell component in the generation of MCs for biomedical applications, deeper inside on the generation of higher quantities of MCs was performed by using the condensed tannin species present in *Mimosa* bark, *i.e.*, *Mim*T. Therefore, *Mim*T-MCs were generated in 5 mL scale using a biphasic system at 1:1 ratio of *Mim*T aqueous solution (pH~12) and olive oil. This system was magnetically stirred for five minutes and then treated by ultrasonication.

- ***NP-MCs generation***

- Preparation of aqueous NP solution : aqueous NP solution was prepared by dissolving a known amount of NPs in distilled H_2O. Thereafter, the pH of this aqueous solution was measure and according to the desired pH an aqueous 1 N NaOH or 1 N NaCl solution was added.

- Preparation of emulsion and NP-MCs generation: according to the desired ratio of the starting mixture a portion of the aqueous natural polyphenol (NP) solution were mixed with a portion of olive oil. This biphasic system was stirred magnetically for 5 min. Using a Branson Digital Sonifier Model 450L (Ultrasonic Corporation) equipped with a 20 kHz Branson probe ending in a sonication tip, the mixture was then

sonicated at room temperature with a power of 160 W (40% amplitude) for the desired time.

- ***Separation of the NP-MCs:*** the separation of the NP-MCs from the balk solvent was realised by centrifugation of the sonicated sample at 5000 rpm for 15 min. The lower part corresponding to the residual emulsion made of the natural polyphenol solution and the organic phase was removed, and the whitish foamy supernatant consisting in the NP-MCs was washed. This washing consisted of re-suspending the NP-MCs in 1000 µL of distilled H_2O, before repeating the separation step as previously described. This washing was performed at least twice in order to obtain the 'concentrated' NP-MCs.

- ***Optical microscopy analysis and statistical analysis of NP-MCs***

- ***Sample preparation:*** 10 µL of the generated 'concentrated' NP-MCs were added in 990 µL of distilled H_2O to form a suspension of NP-MCs. Five microliters of this suspension were transferred on a microscope carrier glass slide and covered with a coverslip prior to microscopy analysis. In case the capsules overlap to a large extend, the sample should be further diluted, prior to analysis.

- ***Analysis:*** A Zeiss Axio Scope A1 microscope was used for the image analysis. All images were obtained with 100 X objective lens magnification. For optimizing the exploitation of the 100 x lens, a drop of mineral oil was placed on the coverslip prior the analysis to function as an optical bridge.

- ***Processing:*** The pictures from the microscope were processed by using the image analysis software ImageJ in combination with a Microsoft Office Excel based analyses for generating statistical data for the NP-MC samples. These statistical data are basically the NP-MCs mean diameter, the minimum and maximum diameters, the NP-MCs number, the polydispersity index (PDI) of the NP-MCs diameter calculated according to the formula σ/μ (where σ is the 'standard' deviation and μ the mean diameter of the NP-MCs in the sample under analysis), and the stability index, which is the ratio between the number of NP-MCs after each experimental condition and the 'standard' conditions. Therefore, this quantitative characterisation of the microcapsules is referred to as the microcapsule's analysis 'standard' procedure.

The different screenings were performed on the basis of statistical key figures (10 µL 'concentrated' NP-MCs at pH~7 in 990 µL distilled H_2O prior to analysis, RT and 1 atm if not stated otherwise). Three images were

chosen for the software-based ImageJ analysis. Error analysis was performed according to this protocol using ten different samples. A comparison between a manual counting and an ImageJ software-based counting has been accomplished, screening different settings in the software. Based on these various evaluations of the quality of the computer-based analysis, an average error of the diameter and the number of MCs per millilitre, 0.06 µm and 0.15 $x10^{12}$ NP-MCs / mL, respectively, were established.

- ***High Performance - Liquid Chromatography (HPLC)***

- *Sample preparation*: approximately 1.0 mg of analyte were dissolved in 1 mL of distilled water and filtered over a 0.20 µm syringe filter prior to injection. By mean of a sample loop, aliquots of 20 µL of the filtered analyte-solutions were analysed at a time.

- *Measurement:* HPLC-analyses were performed using a Shimadzu instrument consisting of a controller unit (CBM-20A), a pumping unit (LC 20AT) equipped with a 20 µL sample loop, a degasser unit (DGU-20A3), a column oven (CTO-20AC), a diode array detector (SPD-M20A), and a refractive index detector (RID-10A); the instrumental set-up was controlled using the Shimadzu LabSolution software package (Version 5.92). A Zorbax C18 analytical column (4.6 (i.d) × 30mm) was connected for analyse. HPLC-grade Acetonitrile water, containing 0.01% of formic acid were used as mobile phase at a 1:2 ratio. The final analyses were performed using the intensities of the UV signal at λ = 280 nm.

II. Results

The variation of the NP concentration was motivated by the fact that failed experiments called our attention about a maximum concentration of NP for successful NP-MC generation, and the outcomes strongly vary as function of NP concentration. In light of this, the suitable concentration of NP was normalised to 0.05 mmol/mL of the total phenolic OH group of NPs.

Furthermore, for generating higher quantities of capsules as needed in the pharmaceutical applications, the previous procedure has been re-visited and optimized by systematically varying the parameters of the starting mixture as well as the ultrasonicator instrument setup. Results of this screening have been reported in Table 1 and Table 2.

Table 1: Representative results[a] of the optimized scalable MC production

Entry	pH[b]	Power [W]	Time [mn]	System[c]	Mean Ø [µm]	Min-Max Ø [µm]	PDI	Conc.[d] [10^{12}MCs/mL]	Exp. Yield[e] [mL]
1	11	40	1	10:1	2.26	0.31-5.95	0.6	0.55	0.2*
2	11	40	10	10:1	1.26	0.31-3.35	0.4	2.64	0.2*
3	11	160	1	10:1	1.25	0.31-3.34	0.4	3.65	0.2*
4	11	160	10	10:1	1.14	0.30-4.04	0.4	4.82	0.2*
5	11	160	10	10:1	1.27	0.30-3.10	0.5	5.79	0.2*
6	12	160	10	1:1	1.43	0.24-2.36	0.2	6.47	0.6*
7	12	160	10	1:1	1.75	0.17-4.12	0.3	6.89	0.6*
8[c]	12	160	10	1:1	1.43	0.24-2.36	0.2	6.47	0.6*
9	12	200	30	1:1	1.43	0.38-2.43	0.2	3.10	4.0+
10	12	300	30	1:1	2.08	0.21-3.84	0.4	3.76	4.5+
11	12	200	30	1:1	1.33	0.30-3.50	0.4	6.25	4.0+
12	12	300	30	1:1	1.58	0.24-3.69	0.4	3.92	3.5+

a: the system was magnetically stirred for five minutes (except entries 5&7) and then treated by ultra-sonication. b: the pH of the aqueous phase; c: the biphasic system ratio of NPs aqueous solution and olive oil; d: concentration of MCs expressed in number of MCs per millilitre [10^{12}MCs/mL]; e: the volume of the 'concentrated' MCs obtained actually at the end of the experiment. This volume was evaluated in a calibrated Eppendorf, and compared to two other Eppendorfs containing a dye solution with a known volume; *MCs were generated in 1 mL scale; + MCs were generated in 5 mL scale.

Specifically, the most relevant outcomes of the characteristic statistical quantitative key numbers of the *Am*T-MCs are reported in Table 2.

Table 2 : Representative results[a] of the optimized scalable *Am*T-MC production

Entry	Aqueous *Am*T solution pH	ratio of the system[b]	mean Ø [µm]	min-max Ø [µm]	PDI	conc. [10^{12}MCs/mL]	exp. yield TMCs [mL][c]
1[d]	5.0	10:1	2.26	0.42-5.83	0.4	0.014	0.01
2	4.5	10:1	1.34	0.25-3.74	0.6	0.13	0.04
3	4.5	1:1	0.98	0.31-3.95	0.7	0.35	0.02
4	7.4	1:1	1.37	0.28-3.64	0.6	0.81	0.10
5	9.5	1:1	0.94	0.28-2.98	0.7	1.90	0.45
6	10.5	1:1	1.44	0.26-3.58	0.4	2.14	0.50
7	11.5	1:1	1.40	0.26-3.83	0.6	3.66	0.50
8	~12	1:1	1.28	0.29-3.63	0.5	6.64	0.50

a: the system was magnetically stirred for five minutes and then treated by ultra-sonication at 160 W for ten minutes; b: the biphasic system ratio of *Am*T aqueous solution and olive oil; c: the volume of the 'concentrated' MCs obtained actually at the end of the experiment. This volume was evaluated in a calibrated Eppendorf, and compared to two other Eppendofs containing a dye solution with a known volume; d: generation of MCs using literature-conditions.

After the rough screening with many NPs in the generation of the NP-MCs reported in table 1 and table 2, with respect to the pharmaceutical applications of MCs, a more inside screening has been performed by using *Mim*T. In this process mainly, the time and the power of the ultra-sonication was systematically varied as reported in Table 3.

Table 3: *Mim*T-MCs[a] generated by screening the time and the power of the ultra-sonication

Entry	Power [W]	Time [nm]	Mean Ø [µm]	Min-Max Ø [µm]	Range[d] [µm]	PDI	Conc. [10^{12}MCs/mL]	Exp. Yield[b] [mL]
1	40	1	1.76	0.53 – 3.08	2.55	0.37	0.296	3.0
2	100	1	1.12	0.48 – 1.95	1.42	0.40	0.567	4.5
3	200	1	2.72	1.39 – 4.15	2.76	0.25	0.172	3.0
4	300	1	2.85	0.85 – 4.07	3.22	0.19	0.234	3.0
5	360	1	2.06	0.90 – 3.02	2.12	0.19	0.333	2.5
6	40	10	1.12	0.36 – 2.20	1.84	0.40	0.690	6.0
7	100	10	1.17	0.53 – 2.10	1.57	0.30	0.567	6.0
8	200	10	1.96	0.49 – 3.71	3.22	0.39	0.308	2.5
9	300	10	2.72	0.85 – 4.90	4.05	0.34	0.246	2.0
10	360	10	1.99	0.44 – 5.32	4.88	0.65	0.185	2.0
11	40	30	1.76	0.40 – 2.78	2.38	0.38	0.739	6.0
12	100	30	2.20	0.62 – 4.20	3.58	0.55	0.370	2.5
13	200	30	2.71	0.72 – 5.93	5.21	0.45	0.308	2.5
14	300	30	2.72	0.89 – 5.91	5.02	0.61	0.271	2.0
15	360	30	2.27	0.46 – 4.57	4.11	0.35	0.542	1.9
16	40	60	1.16	0.55 – 1.98	1.43	0.23	1.37	4.5
17	100	60	1.96	0.91 – 3.57	2.66	0.32	0.246	3.0
18	200	60	1.39	0.60 – 2.61	2.01	0.28	0.480	1.8
19	300	60	2.65	0.91 – 5.02	4.11	0.31	0.271	2.0
20	360	60	1.43	0.31 – 2.91	2.60	0.31	4.23	2.0

a: *Mim*T-MCs were generated in 5 mL scale using a biphasic system at 1:1 ratio of *Mim*T aqueous solution (pH 12) and olive oil. This system was magnetically stirred for five minutes and then treated by ultrasonication; b: the volume of the ‘concentrated’ MCs obtained actually at the end of the experiment. This volume was evaluated in a calibrated Eppendorf, and compared to two other Eppendorfs containing a dye solution with a known volume. c: statistical yield; d: maximum-minimum diameter; e: polydispersity index

Subsequently, the results of the generation of MCs have been thoroughly analysed using the two-factor without replication analysis of variance (ANOVA). The ANOVA of the statistical characteristics of the *Mim*T-MCs reported in Table 3 leads to the following conclusions.

Analysis of the experimental yield: there is a high significant variance at the level of 99%, $F_{0.01}(3,4) = 5.85 > F.crit = 5.4$ and p-value = $0.008 < 0.01$, that the ultrasonication power (w) affects the experimental yield volume of *Mim*T-MCs. Whereas, there is no significant variance even at the level of 90%, $F_{0.1}(3,4) = 0.92 < F.crit = 2.60$ and p-value = $0.46 > 0.1$ that the time of ultrasonication influences the experimental yield volume of *Mim*T-MCs.

Analysis of the MC concentration*:* $F_{0.1}(3,4) = 1.06 < F.crit = 2.48$ and p-value = $0.42 < 0.1$, and$F_{0.05}(3,4) = 1.50 < F.crit = 2.60$ and p-value = $0.46 > 0.1$ of the power and the time of ultrasonication, respectively. Therefore, there is no significant variance at the level of 90%, that neither the power nor the time of ultrasonication affect the concentration of *Mim*T-MCs.

Mean diameter analysis: there is significant variance at the level of 99%, $F_{0.01}(3,4) = 7.18 > F.crit = 5.41$ and p-value = $0.003 < 0.01$, that the ultrasonication power (w) affects the mean diameter of *Mim*T-MCs. Whereas, there is no significant variance at the level of 99%, $F_{0.01}(3,4) = 2.80 < F.crit = 5.95$ and p-value = $0.08 > 0.01$ that the time of ultrasonication influences the mean diameter of *Mim*T-MCs. However, there is significant variance at the level of 90%, $F_{0.1}(3,4) = 2.80 > F.crit = 2.60$ and p-value = $0.08 < 0.1$, that the time of ultrasonication influences the mean diameter of *Mim*T-MCs.

Maximum Diameter analysis: there is significant variance at the level of 95%, $F_{0.05}(3,4) = 5.02 > F.crit = 3.26$ and p-value = $0.01 < 0.05$, that the ultrasonication power (w) affects the maximum diameter of *Mim*T-MCs. Whereas, there is no significant variance at the level of 95%, $F_{0.05}(3,4) = 3.05 < F.crit = 3.49$ and p-value = $0.07 > 0.05$ that the time of ultrasonication influences the mean diameter of *Mim*T-MCs. However, there is significant variance at the level of 90%, $F_{0.1}(3,4) = 2.80 > F.crit = 2.60$ and p-value = $0.07 < 0.1$, that the time of ultrasonication influences the maximum diameter of *Mim*T-MCs.

Diameter Range analysis: $F_{0.1}(3,4) = 4.42 > F.crit = 3.26$ and p-value = $0.02 < 0.05$ and $F_{0.05}(3,4) = 4.30 > F.crit = 3.49$ and p-value = $0.03 < 0.05$ of the power and the time of ultrasonication,

respectively. Therefore, there is a significant variance at the level of 90%, that the power and the time of ultrasonication affect the diameter range value of *Mim*T-MCs.

PDI analysis: there is no significant variance at the level of 90%, $F_{0.1}(3,4) = 0.13 < F.crit = 2.48$ and p-value = $0.97 > 0.1$, that the ultrasonication power (w) affects the PDI of *Mim*T-MCs.. Whereas, there is significant variance at the level of 90%, $F_{0.1}(3,4) = 3.22 < F.crit = 2.61$ and p-value = $0.06 < 0.1$ that the time of ultrasonication influence the PDI of *Mim*T-MCs.

The high pH (~12) value provoked a doubt about the probable hydrolysis of the hydrolysable tannins like EGCG. That is why the HPLC analyses have been performed to have more inside about the hydrolysis products of the EGCG, which should be the Catechin (CAT) and the Gallic Acid (GA). Thus, the starting aqueous EGCG solution at pH 12 as well as the residual aqueous EGCG after the sonochemical process have been analysed *via* HPLC. The HPLC analysis of the hydrolysis products of the EGCG, *i.e.*, the Catechin (CAT) and the Gallic Acid (GA) have been performed and reported in figure 1.

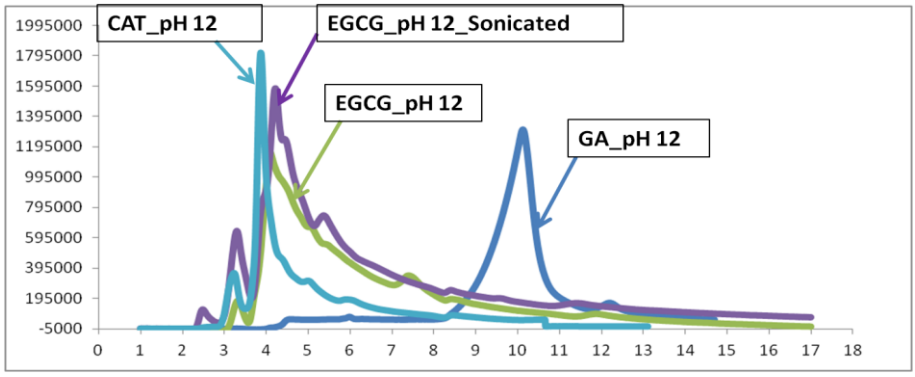

Figure 1: HPLC elution profile of epigallocatechin gallate at pH~12 (EGCG pH 12), sonicated epigallocatechin gallate at pH~12 (EGCG_pH_12_Sonicated); gallic acid at pH~12 (GA_ pH~12) and catechin at pH~12 (CAT at pH~12).

As reported in the figure 1, the traces of the starting aqueous EGCG at pH 12 and the one of the residual aqueous EGCG after the sonochemical process are basically overlapped. On the other hand, the traces of the CAT and the GA, the hydrolysis products of EGCG, are clearly distinct. These results indicate that the high pH does not hydrolyse the EGCG at pH or after the ultrasonication process into CAT and GA.

Table 4 is a recapitulation of the most significant among the eight NPs used in the entire optimization process. These results indicate a mean diameter from 1.26 to 2.43 µm.

Table 4: Set of possible MCs[a] generated using 'standard' natural polyphenols

Entry	NP	Mean Ø [µm]	Min-Max Ø [µm]	PDI	Conc. [10^{12}MCs/mL]	Exp. Yield [mL]
1	**SKL**	**1.55**	**0.31-3.95**	**0.5**	**0.55**	**0.6**
2	**SLS**	**1.26**	**0.31-3.35**	**0.4**	**7.17**	**0.5**
3	***Am*T**	**1.28**	**0.29-3.64**	**0.4**	**6.64**	**0.4**
4	***AmS*T**	**1.44**	**0.30-4.04**	**0.4**	**6.97**	**0.4**
5	**TA**	**2.05**	**0.90-3.78**	**0.4**	**1.15**	**0.4**
6	**CAT**	**2.07**	**0.58–4.23**	**0.5**	**3.05**	**0.5**
7	**EGCG**	**2.22**	**0.68–4.23**	**0.5**	**3.09**	**0.5**
8	***Mim*T**	**2.43**	**0.69–4.66**	**0.4**	**2.26**	**0.5**
9	**FITC-*Mim*T**	**1.32**	**0.43-2.84**	**0.3**	**1.11**	**0.4**

MCs were generated using 1:1 mixture of an aqueous 'standard' natural polyphenols or folate-decorated tannins solution (0.5% w/v, normalised to approximately 0.05 mmol/mL of the total phenolic OH group) at pH~12 and the oily phase, first magnetically stirred for five minutes and subsequently treated by ultrasonication at 160 W (40% amplitude) for ten minutes.

III. Discussion

When approaching the design of MCs, key issues such as biocompatibility, stability, tendency to self-aggregate as well as amphiphilicity need consideration. Therefore, the choice of NPs as MC shell components was based on their overall beneficial characteristic in terms of chemical reactivity and health benefits. Such intrinsic NP properties indicate potential for protecting a sensitive loaded active from oxidative stress and degradation, and to enhance microencapsulated active by synergy.

Some studies had shown that there is no activation towards a crosslinking between polyphenols upon shell formation that might have been induced by the violent ultrasonication conditions (9,10,16,17). Since, only the local up concentrating of polyphenols at the oil-water

interface, and their subsequent advantageous arrangement for favouring efficient stacking between them, seem to be most important, any physical means imposing this kind of stress on the system should be capable of inducing MC formation.

The results of this screening reported in Table 1 and Table 2 led to the assumption that pH is eventually very crucial in inefficient MC formation and not for adjusting solubility as reported before (10,14,16). It has been also noticed that the current standard protocol (14) used a large excess of the NPs. Therefore, 'wasting' NP components was not acceptable anymore. The water/oil phase ratio was also re-visited. In addition, suitable ultrasonication power and time, eventually, could improve polydispersity index (PDI), as a survey of achieved MC systems indicated (16,18–21).

Softwood kraft lignin (SKL) was used first to optimize the conditions of the generation of MCs using the ultrasound assisted method. SKL is particularly cost effective as well as available in industrial quantities (22), and SKL-MCs are reported in scientific literature (14,15,23,24). Table 1. reports the outcomes of the optimization in terms of characteristic statistical quantitative key numbers of the generated SKL-MCs by varying essentially the time and power of the ultrasonication as well as the ratio of aqueous SKL solution and olive oil. Theses characteristic statistical quantitative key numbers are namely the mean diameter as well the minimum and maximum diameters expressed in micrometre, the PDI, the concentration of MCs expressed in number of MCs per millilitre and the experimental yield of the isolated MCs in millilitre. The system was always magnetically stirred for five minutes and then treated by ultrasonication at 160 W for ten minutes. This stirring was aimed to trigger the generation of a perfect emulsion before the ultasonication treatment.

Next, *Acacia mearnsii* bark extract (*Am*T) as exemplary condensed tannin was used to generate *Am*T-MCs by varying essentially the value of pH of the aqueous *Am*T solution and the ratio of aqueous *Am*T solution and olive oil. The system was always magnetically stirred for five minutes and then treated by ultrasonication at 160 W for ten minutes. This stirring like previously stated was aimed to trigger the generation of a perfect emulsion before the ultrasonication treatment.

In addition, based on the primary results obtained in the generation of SKL-MCs as well as *Am*T-MCs, and the beneficial properties of tannins with respect to a function as shell component in the generation of MCs

for biomedical applications, more investigation on the generation of higher quantities of MCs was performed by using the condensed tannin species present in *Mimosa* bark, *i.e.*, *Mim*T (Table 3). The actual use of *Mim*T instead of *Am*T was due to pratical reasons, which is basically impurity issues. In fact, the quantitative ^{31}P NMR spectra reveal the presence of more aliphatic carbohydrate OH-group in *Am*T with respect to *Mim*T, meaning that *Am*T is less pure than *Mim*T.

This approach results in a five-fold increase of volume of isolated and 'concentrated', *i.e.*, densely packed NP-MCs. Most noteworthy, using these optimised conditions, it was possible to process and isolate microcapsules from lignins like SKL, softwood kraft lignin and SLS, softwood lignosofonate; and, more importantly also from tannins like *Am*T, *Acacia mearnsii* bark extract; *AmS*T, *Acacia mearnsii* sulfited tannin; *Mim*T, *Mimosa* tannin; EGCG, epigallocatechin gallate (green tea); TA, tannic acid; CAT, catechin; FITC-*Mim*T, *Mimosa* tannin functionalised with fluorescein isothiocyanate. It is worthy to notice that from previous study it was possible to generate only nanoemulsions via the ultrasound assisted method using EGCG and TA (9).

The HPLC elution profile of the aqueous solution (figure 1) of EGCG at pH~12 before and after the ultrasound treatment do not indicate the presence of gallic acid (GA) and catechin (CAT) as potential products of the hydrolysis of EGCG. It can thus be stated that there is no significant hydrolysis of hydrolysable species using the optimized conditions during the MC formation process. Therefore, the pH (~12) can be considered as one of the most important criteria to fulfil for obtaining the optimum conditions for the generation of the MCs.

Conclusions

For generating higher quantities of MCs a previously reported procedure has been re-visited for optimization. In fact, a 1:1 mixture of an aqueous NP solution (0.5% w/v) at pH~12 and the oily phase, first magnetically stirred for five minutes and subsequently treated by ultra-sonication at 160 W (40% amplitude) for ten minutes, was found to be the most efficient set-up for the generation of NP-MCs using sonochemical method.

It is worth noting that during this work, a centrifuge having 12.000 rpm maximum and an optical microscope have been used as separation and characterization instruments of the generated MCs, respectively. The

MCs fraction was then separated and statistically characterized in mean diameter, minimum and maximum diameters, polydispersity index (PDI) and concentration. However, a more elaborate system of separation and a better microscopic analysis will eventually allow in a subsequent work the separation and further analysis of the nanocapsule fractions.

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