Optimization of silver nanoparticles synthesis by chemical reduction to enhance SERS quantitative performances: early characterization using the quality by design approach

Julie Horne[1*], Charlotte De Bleye[1], Pierre Lebrun[2], Kevser Kemik[1], Thomas Van Laethem[1], Pierre-Yves Sacré[1], Philippe Hubert[1], Cédric Hubert[1†], Eric Ziemons[1†]

[1] *University of Liege (ULiege), CIRM, ViBra-Sante HUB, Laboratory of Pharmaceutical Analytical Chemistry, Department of Pharmacy, Liege, Belgium*

[2] *PharmaLex Belgium, Mont-St-Guibert, Belgium*

[*] *Correspondence: julie.horne@uliege.be*

[†] *These authors contributed equally to this work*

Abstract

Surface-enhanced Raman scattering (SERS) is a vibrational widely used technique thanks to its multiple advantages such as its high specificity and sensitivity. The Raman signal exaltation comes from the use of metallic nanoparticles (Nps) acting as antennas by amplifying the Raman scattering. Controlling the Nps synthesis is a major point for the implementation of SERS in routine analysis and especially in quantitative applications. Effectively, nature, size and shape of these Nps considerably influence the SERS response intensity and repeatability. The Lee-Meisel protocol is the most common synthesis route used by the SERS community due to the low cost, rapidity and ease of manufacturing. However, this process leads to a significant heterogeneity in terms of particle size and shape. In this context, this study aimed to synthesize repeatable and homogeneous silver nanoparticles (AgNps) by chemical reduction. The Quality by Design strategy from quality target product profile to early characterization design was considered to optimize this reaction. The first step of this strategy aimed to highlight critical parameters by the means of an early characterization design. Based on an Ishikawa diagram, five process parameters were studied: the reaction volume as categorical variable and the temperature, the time of reaction, the trisodium citrate concentration and pH as continuous variables. A D-Optimal design of 35 conditions was performed. Three critical quality attributes were selected to maximize the SERS intensity, minimize the variation coefficient on SERS intensities and the polydispersity index of the AgNps. Considering these factors, it appeared that concentration, pH and time of reaction were identified as having a critical impact on the Nps formation and can then be considered for the further optimization step.

Keywords: Surface-enhanced Raman scattering (SERS) - Silver nanoparticles synthesis - Screening design - Quality by Design (QbD) - Chemical reduction

1. Introduction

Nowadays, surface-enhanced Raman scattering (SERS) is a vibrational technique widely used in various domains taking account of its numerous advantages. Among them, the Raman's benefits like the specificity, rapidity and the possibility of real-time analysis are conserved [1,2]. But, the major advantage of this technique is to get around the major Raman limitation by exalting the Raman scattering by a factor from 10^3 to 10^9 [3]. For all these reasons, SERS is used for various applications in several fields. For example, environmental analyses can be driven for the identification of some contaminants or the detection of toxic elements such as arsenic in water [4]. SERS is also used to detect pesticides, toxins or pathogens in food [5,6]. This technique is widely developed in the biological and pharmaceutical domains with notably intracellular analyses [7], in vivo imaging [8] but also to study proteins and lipids [9]. Research of impurities or degradation of some medicines or raw materials is also allowed [10,11]. However, routine analyses with this technique are poorly documented in the literature. This is consequent from the considerable variability of the measured signal [12]. Indeed, a lack of repeatability of SERS signals has already been discussed [13,14]. That can be explained by the heterogeneity of SERS substrates in terms of shape and size. Generally, these substrates are separated into three classes: suspensions of metallic nanoparticles (Nps), Nps deposited onto a solid substrate and solid substrates containing a nanostructure. Several elements can influence the SERS signal including the nature, shape and size of the substrate. However, the analyte itself has also an impact on the SERS response intensity [15]. For all these reasons, controlling the Nps synthesis is essential to obtain a more reproducible signal and to allow SERS quantitative application development. Indeed, if the substrates are not repeatable, the signal cannot be repeatable. An increasing number of researchers work to create repeatable substrates [14]. However, most of the time, techniques giving homogeneous Nps are expensive or difficult to implement [14]. The metallic nanoparticles in suspension remain the substrate the most used by the SERS community. They are usually synthesized by chemical reduction or physical preparation such as laser ablation or lithography [16]. Biological methods are also developed but present little success for SERS analyses due to the potential presence of contaminants and the lower control of shape and size of Nps [17]. Gold and silver are the two main metals used as exaltation agent [3,11]. On the market, commercialized Nps are available giving more guarantee about the batch-to-batch repeatability. However, their matrix composition is often unknown. That may cause an issue for the development of new analysis methods when biological or pharmaceutical samples are considered. Few articles discussed the batch-to-batch

homogeneity and repeatability of synthesized Nps [18]. This is the reason why this study was focused on the optimization of a low-cost and easy to implement way based on a colloidal suspension obtained by chemical reduction. More precisely, this work was dedicated to silver nanoparticles (AgNps) suspensions because they are the most employed as SERS substrates and they also present a high exaltation factor [19].

Among the different synthesis processes, the protocol selected as a starting point for the optimization of AgNps by chemical reduction is the protocol described by Lee-Meisel [20]. This old well-known process continues to be widely used by the SERS community [21]. This reaction consists in a reduction of silver nitrate by trisodium citrate under boiling. Citrate plays the double role of reducing agent and stabilizing agent [21]. Despite the Lee-Meisel process has several advantages such as rapidity, ease of implementation and low cost, its major limitation is the heterogeneity of obtained Nps both in terms of size and shape [13,21]. To tackle this issue, an innovative approach based on the Quality by Design (QbD) strategy will be used to synthesize homogeneous and repeatable AgNps. QbD is a development strategy involving design of experiments (DoE) to maximize information obtained with a minimum of experiments [22]. Moreover, with DoE, all the multivariate factors are simultaneously modified to account for their influence on each other [23]. This approach allows a modeling of all conditions and, with appropriate statistics, can lead to define a robustness zone for the Nps synthesis process [24].

The QbD strategy used is schematized in Figure 1. The first step consists of defining the quality target product profile (QTPP) describing the intended purpose of the process. Then, the process to be optimized can be selected. Based on a risks assessment, the critical quality attributes (CQAs) allowing to measure this defined goal are selected as well as the process parameters (PPs) associated. CQAs are the attributes that express the quality of the process as defined in the QTPP while PPs are the parameters that may have a critical impact on the process in regards of the CQAs. Based on these, if necessary, an experimental design can be used to determine critical process parameters (CPPs) which have the most influence on the CQAs (early characterization step). Indeed, CPPs influencing the process can then be optimized or controlled to ensure an optimal process is defined. Often, a second experimental design is run to get more precise estimates of the factor effects. Based on this last experimental step, a design space is determined, that highlights an optimal zone associated with a probability of success that determines the combination of process parameters allowing to achieve the intended purpose of the process. Synthesis conditions are then tested and can be validated and used. However, this process needs to be regularly controlled to guarantee its quality thanks a well-defined control

strategy. This is why, QbD can be considered as a continual improvement strategy [25]. This article is focalized on the first part of the QbD strategy: from the QTPP to the early characterization design and was notably based on the *a-priori* knowledge from the laboratory xpertise and preliminary tests (Figure 1, steps 1 to 5) [11,24,26]. All these steps aimed to finally optimize the synthesis of AgNps to obtain reproducible SERS substrates giving more perspectives to quantitative applications of this vibrational technique in the pharmaceutical and biomedical fields.

2. Materials and methods

2.1. Chemicals and reagents

Silver nitrate and potassium chloride were purchased from VWR (Pennsylvanie, USA), trisodium citrate from Acros Organics (Geel, Belgium) and violet crystal came from TCI Europe N.V. (Zwijndrecht, Belgium). All solutions and dilutions were made using MilliQ water (18.2 MΩ.cm, Milli-Q Plus 185, Millipore, Burlington, USA). Formic acid and NaOH were purchased from VWR (Pennsylvanie, USA) and TCI Europe N.V. (Zwijndrecht, Belgium), respectively.

2.2. Silver nanoparticles synthesis

For the chemical reductions, all steps were made far of light to avoid the silver oxidation. Silver nitrate ($AgNO_3$) solution of $1x10^{-3}$ M was placed in a 3-necks flask onto a Drysyn heating bath (Asynt, England) with a temperature set on 150 °C or 180 °C (according to the early characterization design). Temperature was measured in the oil heating bath placed under the reaction flask. Once the solution was boiling, 5.0 mL of citrate solution were added at 5 mL/min rate by the means of a dosing device (Dosimat, Metrohm AG, Switzerland) directly by an empty neck of the 3-necks flask. Two concentrations of citrate solution were tested (0.02 and 0.10 M) depending on the early characterization design defined. The pH of this reducing solution was adjusted with sodium hydroxide 0.1 mol/L or formic acid 99%. Reaction was stopped after the time defined in the design and the resulting suspension was cooled down at room temperature. The AgNps suspensions were then characterized and conserved in the dark at 4 °C. Table 1 illustrates the synthesis protocol step by step.

2.3. AgNps characterization

Different techniques were used for the SERS substrates characterization. The pH measurements were performed using a Seven Easy Mettler Toledo pH-meter® (Mettler-Toledo, Ohio, USA). Mean diameter of AgNps with the corresponding polydispersity index (PDI) and the zeta potential were obtained respectively by a Malvern Panalytical ZetaSizer Nano ZS DLS and an electrophoretic light scattering (ELS) equipment (Malvern Panalytical, Malvern, United Kingdom). The laser wavelength selected was 632.8 nm. For these analyses, a specific cuvette (Malvern Panalytical, Malvern, United Kingdom) containing the ultrasonicated and 10 times diluted suspension was used.

TEM investigations were completed with a Jeol JEM-1400 TEM (Jeol, Tokyo, Japan) at 80 kV after depositing a drop of the suspension onto a formvar/carbon coated copper grid (Jeol, Tokyo, Japan). The maximum magnification for this study was set on 40 000. ImageJ® 1.53k (Wayne Rasband and contributors, National Institutes of health, USA) was used to estimate a mean size of AgNps based on the TEM pictures.

2.4. SERS analyses

SERS analyses were performed on a LabRAM HR Evolution Raman microscope (Horiba Jobin-Yvon, Lyon, France) with a 785 nm laser (100 mW on the sample) and a 300 grooves/mm grating, a two-dimensional electron multiplying charge-coupled device (EMCCD) detector (1600 x 200 pixels sensor) and a 50x long working distance (LWD) objective with a confocal slit-hole of 200 µm. The acquisition time was set on 7 s. Three acquisitions were taken per sample with three accumulations.

For each AgNps synthesis, three independent samples were prepared with three different aggregating agent concentrations (potassium chloride - KCl). For the SERS sample preparation, 400 µL of AgNps were mixed for 10 s with 400 µL of violet crystal ($1x10^{-6}$ M) using a vortex (Reax top, Heidolph, Schwabach, Germany) before adding 100 µL of KCl (0.1, 0.3 or 0.5 M). The resulting solution was mixed for 10 s before being analyzed in triplicate using a 96-well plate.

MatLab® R2020b (The MathWorks, Natick, MA, USA) and PLS_Toolbox 8.9.2 (Eigenvector Research, Inc., Wenatchee, WA, USA) were used for the preprocessing of SERS spectra. This treatment consisted in a baseline correction (automatic Whittaker filter with a lambda of 10 000 and p of 0.01).

2.5. Early characterization design

JMP® Pro 15 software (SAS Institute, Cary, USA) was used for the elaboration and the interpretation of the early characterization design. This design was computed based on the PPs and the CQAs. A D-Optimal design was selected to estimate all main effects, 2-factors interactions, and the quadratic effect of the factor pH^2. This design is built for 5 continuous factors and was separated in 12 orthogonal blocks performed in 12 days for practical reasons (only three suspensions can be synthesized on the same day) and to take into account the day-to-day variability. According to the *a-priori* knowledge, a high response variability was anticipated requiring to multiple experiments. This is the reason why 35 synthesis conditions were required and were presented in the Table S1.

3. Results and discussion

3.1. Quality by Design (QbD) strategy

The first part of this study was devoted to the steps 1 to 5 of Figure 1 of this strategy being the formalization of the QTPP, the selection of the process, the risk assessment, the selection of PPs and CQAs and the realization of an early characterization design to determine the CPPs impacting on the AgNps synthesis. Early characterization design is more involved than a classical screening design but cannot be considered as an optimization design. This plan consists in synthesizing AgNps with different conditions and characterizing them relating to the CQAs. Finally, the interpretation of the characterization plan will determine the CPPs and their range prior to go towards the optimization phase.

3.1.1. Quality target product profile (QTPP)

This project aims to optimize the synthesis of AgNps specifically for SERS quantitative applications. In this context, silver nanoparticles need to be homogeneous and the synthesis process must be repeatable. Indeed, size and shape of these Nps need to be as identical as possible within the same suspension as well as between different batches to harmonize the detected signal intensity. No specific size was expected, only the repeatability and the homogeneity are aimed.

Consequently, the QTPP can be defined as “homogeneous AgNps in size and shape without a specific size requested using a chemical reduction process and obtained in a repeatable manner for intra- and inter-batches”.

3.1.2. Process selection

Based on our *a-priori* knowledge, chemical reduction based on the Lee-Meisel protocol was selected as synthesis process. This widely used process allows obtaining AgNps in “friendly” conditions but need to be optimized to form homogeneous and repeatable SERS substrates. This protocol was selected for its consequent use by the SERS community, its low cost, its simplicity of implementation and its rapidity.

3.1.3. Risk assessment and PPs determination

Process parameters (PPs) and material attributes can have an impact on the goal of the study. An assessment of the risks was realized by the means of an Ishikawa diagram, as illustrated by Figure 2. This tool is frequently used with the QbD concept and represents an overview of different parameters that are interesting to consider for the early characterization design [26]. Concentration of trisodium citrate seemed to have an impact on the shape of the Nps formed during the process but also on their stability [27]. This effect can be explained by the fact that citrate interacts with the Nps surface and plays the role of stabilizing agent [28]. By modifying the pH of the citrate solution, the shape and the dispersity of particles can also be modified [29]. In addition, reaction time and temperature have also an impact on the size distribution of the Nps synthesized [30]. In this framework and based on our *a-priori* knowledge, the five most influencing parameters were selected as highlighted in Figure 2. Due to the selection of five parameters, a full factorial design with a total of 72 experiments was not feasible. Therefore, the strategy is focused on an early characterization design. Moreover, a high response variability was anticipated increasing the number of experiments. Material attributes have a minor probability to influence the synthesized Nps and were not selected as PPs.

These parameters are summarized in Table 2. Three reaction volumes were considered as categorical variable while other parameters were continuous variables with two levels except for the pH that counted three levels. Indeed, pH involves according to a sigmoidal distribution and need three points to evaluate this effect within the investigated range. Depending on commercialized gauged flasks and our *a-priori* knowledge, reaction volume was selected as categorical variable. In the protocol described by Lee and Meisel, the reaction time is one hour

after the addition of citrate solution. Therefore, a lower (30 min) and a higher (120 min) reaction time were chosen. Finally, a lower temperature (150 °C) than the initial protocol (180 °C) was selected as lower limit while the initial value was the higher for this design. The interval of citrate concentration was adapted from the literature and the laboratory expertise [11,27,29,31].

3.1.4. CQAs determination

The main responses of this early characterization design were represented by the CQAs. Based on the risk assessment, our *a-priori* knowledge and according to the final aim of the optimization of AgNps synthesis, three CQAs were selected with their acceptance criteria. The polydispersity index (PDI) complemented the second CQA because size and shape of Nps influence the SERS signal. If Nps suspension is heterogeneous, the SERS intensity will inevitably vary from one analysis to the other. These values were arbitrarily fixed based on the technique and the model compound used and were presented in the Table 3.

3.1.5. Early characterization design

Each synthesis leads to the production of AgNps which were characterized to determine the CQAs and to obtain more information about their shape and their size. No data was missing during these experiments. Among the different techniques used for the nanoparticles characterization, dynamic light scattering (DLS) was selected in this project to obtain the polydispersity index (PDI) that reflects the Nps dispersity in terms of size. Moreover, with the objective of evaluating the repeatability of the process and the exaltation factor, Nps were considered to perform SERS analyses using a model molecule, the violet crystal.

First, DLS gave the PDI which showed the dispersity in terms of Nps size. When this index tends to zero, population is considered as monodispersed (lower than 0.05) while when it tends to one (higher than 0.7), particles are polydispersed [32]. For this design, obtained results are presented in the Table S1 and are comprised between 0.119 and 0.476. The dispersity of AgNps in terms of size was then considerable and needed to be minimized. This CQA was chosen to obtain homogeneous Nps and then having repeatable SERS signal.

Afterwards, SERS measurements were realized to evaluate two CQAs, the intensity of SERS measurement, which needed to be maximized, but also the associated relative standard deviation (RSD%), which had to be minimized. Concerning signal intensity for a specific band (1170 cm^{-1}) of the violet crystal molecule, larger variations were pointed as shown on Figure 3. The violet crystal concentration selected was equal to $1x10^{-6}$ M to avoid the saturation of the

detector and giving a correct signal to noise ratio. The minimum RSD% obtained was equal to 2.84%. Figure 4 illustrates SERS spectra for the synthesis with the lower RSD% value (1B) and for the one giving the higher SERS intensity (11C). For SERS measurements, an aggregation agent was added to the sample to gently aggregate Nps. This phenomenon allows the creation of "hot-spots", it means that Nps are close to each other and the signal exaltation is tremendous enhanced. For this study, three different concentrations of KCl (0.1, 0.3 and 0.5 M, chosen based on the *a-priori* knowledge) were systematically tested for each colloidal suspension to evaluate their capacity to form hot-spots. Indeed, particles concentration and their properties were not the same for each synthesis. For instance, mean size and specific surface are different. So, AgNps did not have the same capacity to aggregate in a suitable way. The concentration giving an observable response with the lower RSD% value was retained and is reported in the Table S1. Priority goes to the RSD% because it represents the main CQA followed to achieve a repeatable synthesis protocol. Moreover, the KCl concentration was not selected as CQA because its concentration is not predictable. Given that the objective of the early characterization design was to determine the critical character of selected parameters, the KCl concentration was not integrated because it is already known that this parameter is critical. The KCl concentration will be optimized later for the optimal synthesis.

After the CQAs collection, the results of the early characterization design can be interpreted. In most screening studies, experimenters focus on establishing tables of parameters estimates, including a p-value stating if a parameter can be identified as significantly different from 0. Table S2 shows the p-values of each factor for each individual model. However, this approach often fails to link the parameter effect to the QTPP. For this purpose, already at this early stage, it is here advocated to compute the probability to meet or to fail the acceptance criteria for all CQA jointly, hence linking the modeling to the QTPP objective. Figure 5 shows the early characterization design interpretation. Interesting information are highlighted with bright colors. When coloration tends to green, the probability of success for the combined CQAs becomes higher (log10 Defect Rate becomes lower). To the contrary, the more the coloration is red, the less the joint probability of success is high. In this situation, success can be defined as simultaneously a SERS signal intensity superior to 15 000 counts, a RSD% and a PDI inferior to 30% and 0.3, respectively. It is true that 30% for a RSD% remains high but it was in the framework of this experimental design. The objective was to select parameters which have a critical impact on the Nps formation. This value represents a threshold beyond which the results are deemed unacceptable. Corrections and optimizations are possible for values falling below

this value. Thanks to this limit, good and bad results can be distinguished from each other. Most interesting information are pointed out in the green rectangles in the Figure 5. First, the trisodium citrate concentration appeared as a critical parameter on the formation of SERS substrates. A positive effect of the lower concentration was noticed by the presence of more green points in this range. It is the same behavior for the pH, a positive effect with the lower pH values. According to the final objective of this study, those parameters will be studied for the optimization phase. Time of reaction also seems to have an influence on the CQAs. Its impact is less pronounced than the other parameters but a slight positive effect is highlighted for the lower reaction time. Thus, this parameter will also be considered for the optimization design. These conclusions were verified as illustrates Figure S1. Indeed, low pH value associated to the little citrate concentration showed high SERS intensities with a low RSD% and a low PDI as it was observed for the 4A and 10C syntheses. On the contrary, 9C obtained by a high citrate concentration and a pH of 10.4 showed more variability and a weak SERS intensity. Finally, this area of the early characterization design seems to show good results according the selected CQAs. Concentration and pH of trisodium citrate solution and time of reaction will be the critical process parameters that will be considered for the optimization phase.

3.2. Complementary characterization

In parallel with the AgNps characterization for the collection of CQAs, other tools and information were obtained but were not integrated in the design. DLS gives size of Nps which is an interesting tool to be sure that AgNps are in the adequate range for SERS measurements. Interesting size range for Nps for SERS measurements reported is generally from 10 nm to 100 nm [33,34]. However, since no specific size was targeted, this factor was excluded as a CQA. Zeta potential was also measured. It is a good factor to evaluate the stability of the suspension. This stability will be evaluated once the protocol will be optimized. On the other hand, transmission electron microscopy (TEM) was used to visualize AgNps and to evaluate their shape. It is important to consider this factor because the DLS estimates a spherical shape for all Nps measured.

In addition to the PDI, dynamic light scattering offered some interesting information such as the mean size of Nps. The mean size of AgNps synthesized during this early characterization design varied from 25 to 99 nm as reported in the Table S1. This range matched perfectly with the interesting range for SERS analyses (from 10 nm to 100 nm) [34].

Another characteristic could be obtained using this equipment, the zeta potential. The Nps charge was negative for all the syntheses thanks to the citrate stabilization. Zeta potential also informed on the stability of AgNps. The more the zeta potential is high, the more the suspension is stable [35]. As showed in the Table S1, most of the syntheses had a value near 40-45 mV but five of them had value of 25-30 mV. These last will be aggregated faster than the others leading to a flocculation and an absence of SERS response.

On the other hand, microscopy allows the visualization of Nps. Thereby, the shape of these substrates can be directly observed as well as the size distribution. Several syntheses with different PDI were analyzed by TEM to have an idea of their dispersity both in terms of shape and size. Moreover, the more the AgNps are spherical, the more DLS measurement are reliable. Figure 6 shows pictures obtained by TEM for three different PDI reflecting different states of dispersion of Nps. The first batch had a PDI of 0.422 and showed a high dispersity of shape with spheres and rods of multiple sizes. The mean evaluated size based on TEM pictures for this synthesis is 61 nm (n= 191, RSD% of 46%). On the contrary, pictures from a PDI of 0.181 showed more homogeneity of shape and a lower scale of size. However, these Nps cannot be considered as homogeneous with a unique size and shape. The estimated mean size is 41 nm (n= 361, RSD% of 42%). Finally, the presented synthesis with a PDI of 0.322 also contained a lot of different sizes and many different shapes with a mean size of 77 nm (n= 219, RSD% of 31%).

4. Conclusion

In conclusion, this article presents an original strategy applied for the first time for the early characterization of AgNps synthesis by chemical reduction. The main objective of this project is the elaboration of a protocol giving homogeneous and repeatable AgNps to expand the quantitative perspectives of SERS in the pharmaceutical or biomedical fields for example. Indeed, homogeneous SERS substrates are primordial to measure a repeatable SERS signal and to ensure new quantitative studies. If nanoparticles are homogeneous, it can be expected that the enhancement will also be repeatable. The presented results aimed to highlight critical parameters by the help of an early characterization design in order to optimize them by a performing and innovating strategy. The first step of the QbD process was able to succeed and showed encouraging results. The strategy employed calculates a probability of success based on the interaction of all the CQAs. This study showed parameters that have a critical impact on

the AgNps formation to simultaneously maximize the SERS intensity and minimize the RSD% on SERS intensities and the PDI. Among the five process parameters tested, concentration and pH of citrate solution and reaction time have significant impact. It means that these three CPPs will be conserved for the next step of this work consisting in the elaboration of an optimization design. This design will also restrict the ranges of parameters for concentration (0.02 – 0.06 M) and pH (5 – 9) based on the design interpretation. Fixed values for the CQAs will also be more stringent during the optimization design. Finally, QbD is an excellent strategy to robustly optimize synthesis process by considering interaction between parameters while limiting the number of experiments to carry out.

CRediT authorship contribution statement:

Julie Horne: Conceptualization, Methodology, Formal Analysis, Writing - Original Draft Preparation, Writing - Review and Editing. **Charlotte de Bleye**: Conceptualization, Methodology, Writing - Review and Editing, Supervision. **Pierre Lebrun**: Software, Writing - Review and Editing. **Kevser Kemik**: Formal Analysis. **Thomas Van Laethem**: Writing - Review and Editing. **Pierre-Yves Sacré**: Software, Writing - Review and Editing. **Philippe Hubert**: Supervision, Project Administration, Funding Acquisition. **Cédric Hubert**: Conceptualization, Writing – Review and Editing, Supervision, Project Administration. **Eric Ziemons**: Conceptualization, Writing - Review and Editing, Supervision, Project Administration. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: The authors thank Nicolas Thelen (Giga Neurosciences, ULiege, Belgium) for the acquisition of TEM pictures. DLS measurements were possible with the help of Géraldine Piel (Laboratory of Pharmaceutical Technology and Biopharmacy, ULiege, Belgium).

Funding: This research was supported by Walloon Region, thanks to a FRIA grant and by the Leon Fredericq foundation.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the study or in the decision to publish the results.

References:

1. S. Pang, T. Yang, L. He, Review of surface enhanced Raman spectroscopic (SERS) detection of synthetic chemical pesticides, TrAC-Trend. Anal. Chem. 85 (2016) 73–82.
2. R.A. Alvarez-Puebla, L.M. Liz-Marzán, Traps and cages for universal SERS detection, Chem. Soc. Rev. 41(1) (2012) 43–51.

3. J. Cailletaud, C. De Bleye, E. Dumont, P.Y. Sacré, L. Netchacovitch, Y. Gut, M. Boiret, Y.M. Ginot, Ph. Hubert, E. Ziemons, Critical review of surface-enhanced Raman spectroscopy applications in the pharmaceutical field, J. Pharm. Biomed. Anal. 147 (2018) 458–472.
4. J. Hao, M.J. Han, S. Han, X. Meng, T.L. Su, Q.K. Wang, SERS detection of arsenic in water: A review, J. Environ. Sci. 36(V) 2015 152–162.
5. J. Perumal, Y. Wang, A.B.E. Attia, U.S. Dinish, M. Olivo, Towards a point-of-care SERS sensor for biomedical and agri-food analysis applications: A review of recent advancements, Nanoscale 13(2) (2021) 553–580.
6. Z. Lin, L. He, Recent advance in SERS techniques for food safety and quality analysis: a brief review, Curr. Opin. Food Sci. 28 (2019) 82–87.
7. I. Chourpa, F.H. Lei, P. Dubois, M. Manfait, G.D. Sockalingum, Intracellular applications of analytical SERS spectroscopy and multispectral imaging, Chem. Soc. Rev. 37(5) (2008) 993–1000.
8. Z. Du, Y. Qi, J. He, D. Zhong, M. Zhou, Recent advances in applications of nanoparticles in SERS in vivo imaging, Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology 13(2) (2021) 1–16.
9. I. Bruzas, W. Lum, Z. Gorunmez, L. Sagle, Advances in surface-enhanced Raman spectroscopy (SERS) substrates for lipid and protein characterization: Sensing and beyond, Analyst 143(17) (2018) 3990–4008.
10. M.T. Alula, Z.T. Mengesha, E. Mwenesongole, Advances in surface-enhanced Raman spectroscopy for analysis of pharmaceuticals: A review, Vib. Spectrosc. 98(June) (2018) 50–63.
11. C. De Bleye, E. Dumont, E. Rozet, P.Y. Sacré, P.F. Chavez, L. Netchacovitch, G. Piel, Ph. Hubert, E. Ziemons, Determination of 4-aminophenol in a pharmaceutical formulation using surface enhanced Raman scattering: From development to method validation, Talanta 116 (2013) 899–905.
12. M. Berge, A. Dowek, P. Prognon, F.X. Legrand, A. Tfayli, Lê. L. Minh Mai, E. Caudron, Optimization of experimental conditions by surface enhanced Raman Scattering (SERS) spectroscopy with gold nanoparticles suspensions, Spectrochim. Acta - Part A Mol Biomol. Spectrosc. 268 (2022) 120628.
13. J.F. Betz, W.W. Yu, Y. Cheng, I.M. White, G.W. Rubloff, Simple SERS substrates: Powerful, portable, and full of potential, Phys Chem 16(6) (2014) 2224–2239.
14. T. Gao, Y. Wang, K. Wang, X. Zhang, J. Dui, G. Li, S. Lou, S. Zhou, Controlled synthesis of homogeneous ag nanosheet-assembled film for effective SERS substrate, ACS Appl. Mater. Interfaces 5(15) (2013) 7308–7314.
15. Y. Li, J. Zhu, G. Weng, J. Li, T. Xing, J. Zhao, Morphologically controllable AuAg@AuAg yolk-shell nanostars with better plasmonic optical properties, J. Alloys Compd. 844 (2020) 156134.
16. P.A. Mosier-Boss, Review of SERS substrates for chemical sensing, Nanomaterials 7(6) (2017).
17. O. Pryshchepa, P. Pomastowski, B. Buszewski, Silver nanoparticles: Synthesis, investigation techniques, and properties, Adv. In Col. And Inter. Sci. 284 (2020) 102246.

18. C. Muehlethaler, M. Leona, J.R. Lombardi, Towards a validation of surface-enhanced Raman scattering (SERS) for use in forensic science: repeatability and reproducibility experiments, Forensic Sci. Int. 268 (2016) 1–13.
19. M.V. Cañamares, J.V. Garcia-Ramos, S. Sanchez-Cortes, M. Castillejo, M. Oujja, Comparative SERS effectiveness of silver nanoparticles prepared by different methods: A study of the enhancement factor and the interfacial properties, J. Colloid. Interface Sci. 326(1) (2008) 103–109.
20. P.C. Lee, D. Meisel, Adsorption and surface-enhanced Raman of dyes on silver and gold sols, J. Phys. Chem. 86(17) (1982) 3391–3395.
21. B. Dong, N. Xue, G. Mu, M. Wang, Z. Xiao, L. Dai, Z. Wang, D. Huang, H. Qian, W. Chen, Synthesis of monodisperse spherical AgNPs by ultrasound-intensified Lee-Meisel method, and quick evaluation via machine learning, Ultrason. Sonochem. 73 (2021) 105485.
22. S. Orlandini, R. Gotti, S. Furlanetto, Multivariate optimization of capillary electrophoresis methods: A critical review, J. Pharm. Biomed. Anal. 87 (2014) 290–307.
23. P.K. Sahu, N.R. Ramisetti, T. Cecchi, S. Swain, C.S. Patro, J. Panda, An overview of experimental designs in HPLC method development and validation, J. Pharm. Biomed. Anal. 147 (2018) 590–611.
24. R. Deidda, S. Orlandini, Ph. Hubert, C. Hubert, Risk-based approach for method development in pharmaceutical quality control context: A critical review, J. Pharm. Biomed. Anal. 161 (2018) 110–121.
25. European Medicines Agency (EMA), ICH guideline Q8 (R2) on pharmaceutical development. EMA/CHMP/ICH/167068/2004 Committee for Human Medicinal Products, Ema/Chmp/Ich/167068/2004, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002872.pdf%0Ahttps://www.ema.europa.eu/en/documents/scientific-guideline/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-huma (accessed on 25 November 2022).
26. R. Deidda, H.T. Avohou, E. Dumont, C. Hubert, Ph. Hubert, C. De Bleye, E. Ziemons, Application of the analytical quality by design principles to the development of a qualitative surface-enhanced Raman scattering method: A proof of concept, J. Raman Spectrosc. 53(1) (2022) 20–32.
27. D.M. Ledwith, A.M. Whelan, J.M. Kelly, A rapid, straight-forward method for controlling the morphology of stable silver nanoparticles, J. Mater. Chem. 17(23) (2007) 2459–2464.
28. S. Das, K. Bandyopadhyay, M.M. Ghosh, Effect of stabilizer concentration on the size of silver nanoparticles synthesized through chemical route, Inorg. Chem. Commun. 123(August 2020) (2021).
29. X. Dong, X. Ji, H. Wu, L. Zhao, J. Li, W. Yang, Shape control of silver nanoparticles by stepwise citrate reduction, J. Phys. Chem. C. 113(16) (2009) 6573–6576.
30. Y. Wan, Z. Guo, X. Jiang, K. Fang, X. Lu, Y. Zhang, N. Gu, Quasi-spherical silver nanoparticles: Aqueous synthesis and size control by the seed-mediated Lee-Meisel method, J. Colloid Interface Sci. 394(1) (2013) 263–268.

31. C. De Bleye, E. Dumont, C. Hubert, P.Y. Sacré, L. Netchacovitch, P.F. Chavez, Ph. Hubert, E. Ziemons, A simple approach for ultrasensitive detection of bisphenols by multiplexed surface-enhanced Raman scattering, Anal. Chim. Acta. 888 (2015) 118–125.
32. M. Danaei, M. Dehghankhold, S. Ataei, F. Hasanzadeh Davarani, R. Javanmard, A. Dokhani, S. Khorasani, M.R. Mozafari, Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems, Pharmaceutics 10(2) (2018) 1–17.
33. G. Kumari, J. Kandula, C. Narayana, How Far Can We Probe by SERS? J. Phys. Chem. C. 119(34) (2015) 20057–20064.
34. R.X. He, R. Liang, P. Peng, Y. Norman Zhou, Effect of the size of silver nanoparticles on SERS signal enhancement, J. Nanoparticle Res. 19(8) (2017).
35. S. Bhattacharjee, DLS and zeta potential - What they are and what they are not? J. Control Release 235 (2016) 337–351.

Appendices

Figures captions

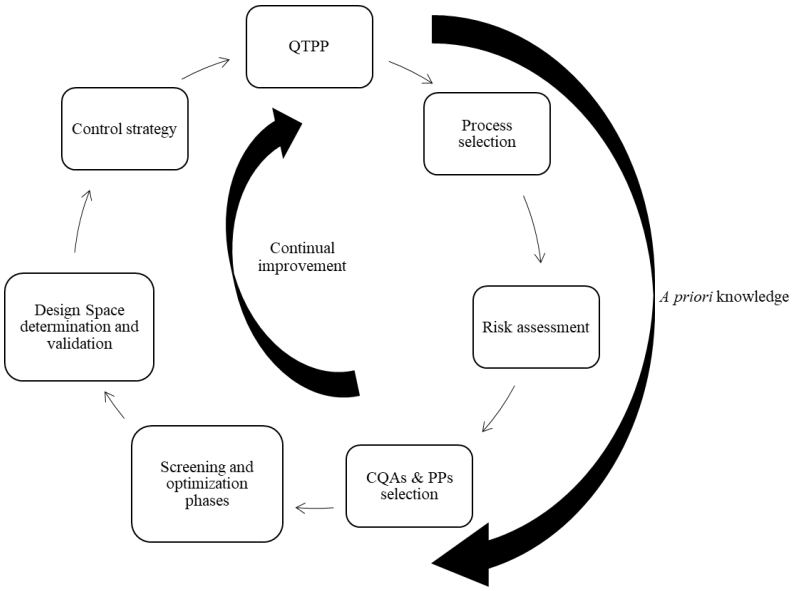


Figure 1: Schematic representation of the Quality by Design (QbD) strategy applied to the synthesis of silver nanoparticles by chemical reduction (QTPP = quality target product profile, PPs = process parameters, CQAs = critical quality attributes)

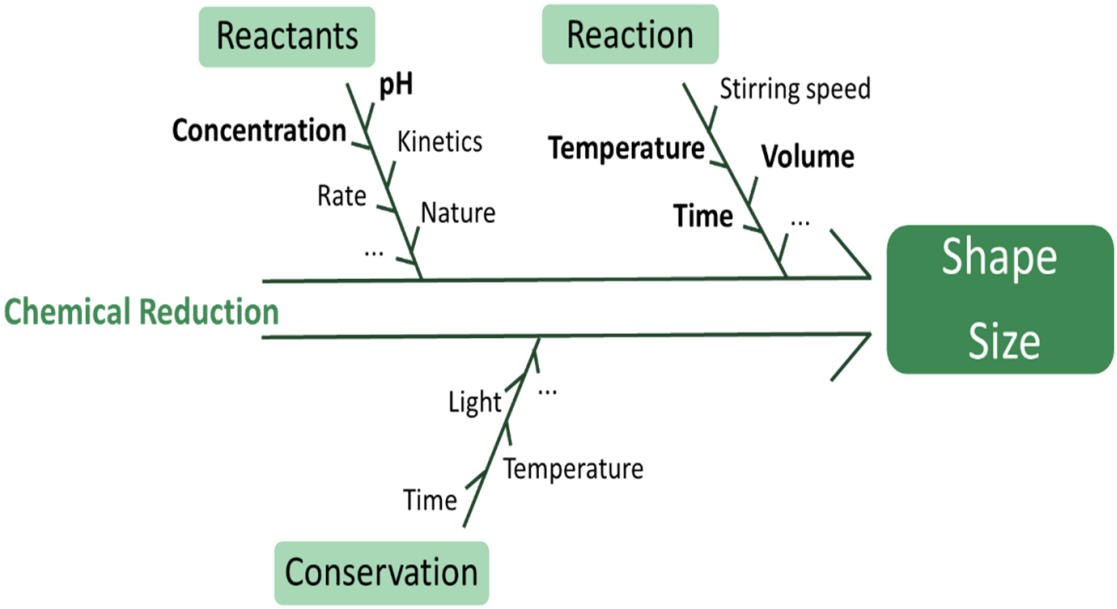


Figure 2: Ishikawa diagram to select process parameters (PPs) to consider for the early characterization design.

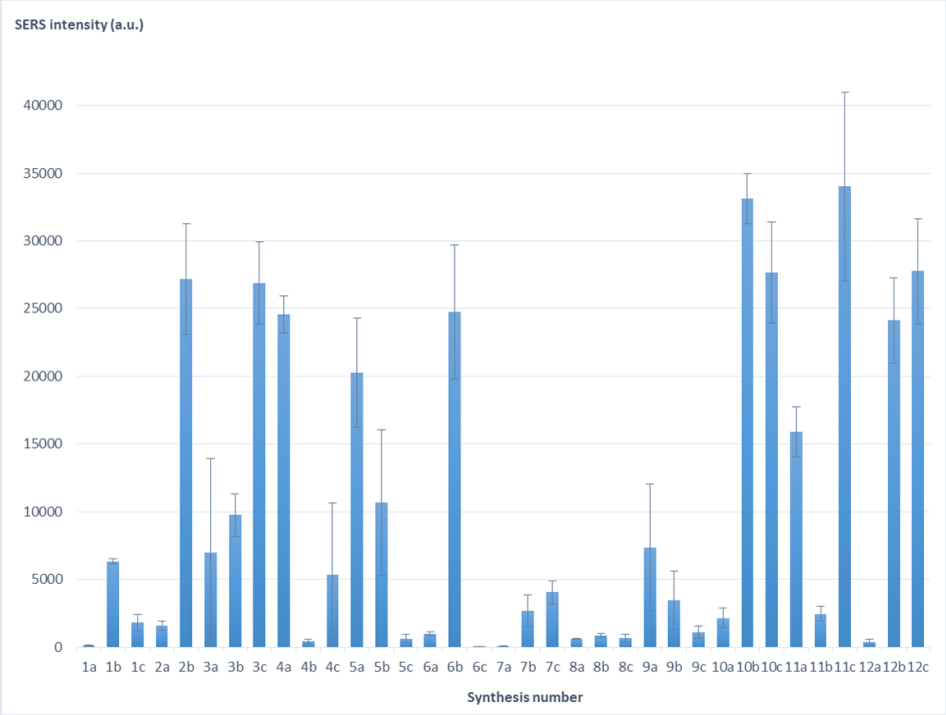
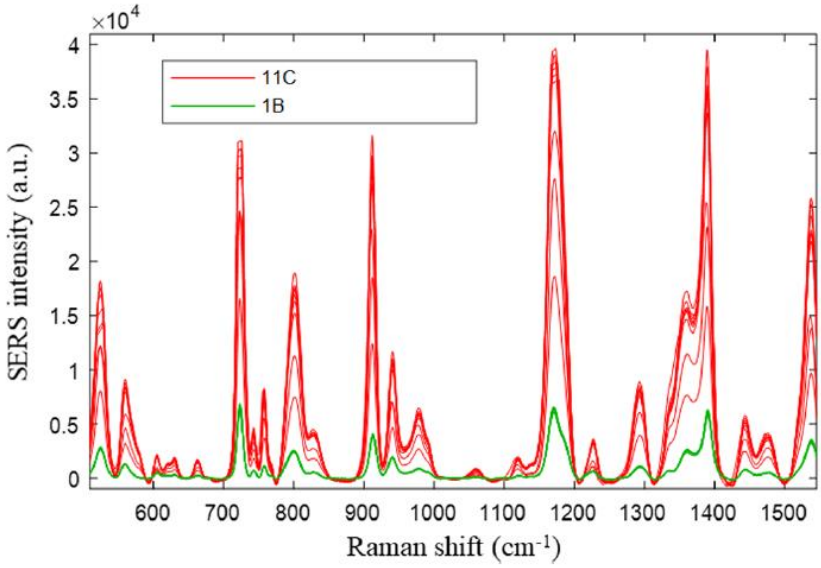
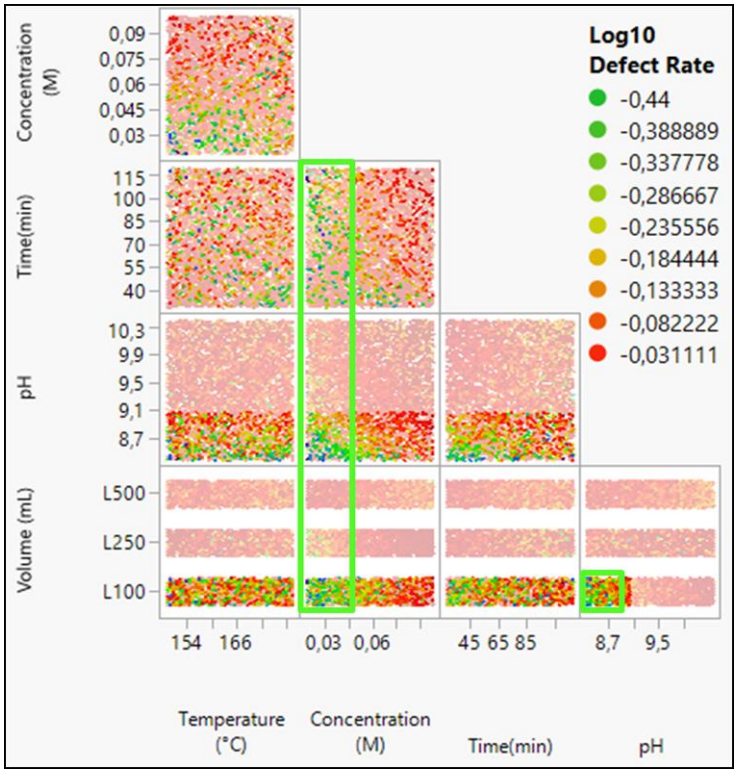


Figure 3: SERS intensity with corresponding variation for each synthesis of the early characterization design (n=9).



504

505 **Figure 4:** SERS spectra examples for 2 conditions of the early characterization design
506 (pretreatment consisted to a baseline correction: automatic Whittaker filter with a lambda of
507 10 000 and p of 0.01, n=9 per condition).



508

509 **Figure 5:** Early characterization design interpretation. The more points are green, the more
510 success probability is high. On the contrary, red points shows low success probability. Green
511 rectangles show interesting effect and critical parameters

Figure 6: Transmission electron microscopy (TEM) pictures with a 20 000 magnification of silver nanoparticles synthesized by chemical reduction during the early characterization design. Several syntheses were observed with different polydispersity index (PDI), (a) Synthesis 7A with a PDI of 0.422; (b) Synthesis 2B with a PDI 0.322; (c) Synthesis 12A with a PDI of 0.181.

Tables

Table 1: Summarize of AgNps synthesis protocol step by step

Step	Fixed parameter	Variable parameter
Concentration of $AgNO_3$ (M)	$1x10^{-3}$	-
Volume of $AgNO_3$ (mL)	-	100 – 500
Concentration of citrate solution (M)	-	0.02 – 0.10
pH of citrate solution	-	8.4 – 10.4
Addition of citrate solution	When $AgNO_3$ was boiling 5.0 mL at 5 mL/min	-
Temperature set for the oil bath during the reaction (°C)	-	150 – 180
Time of reaction (min)	-	30 – 120
End of reaction	Cooled down at room temperature – conserved at 4 °C in the dark	-

Table 2: Selected parameters and levels for the early characterization design

Variable type	Categorical			Continuous		
Level	-1	0	+1	-1	0	+1
Reaction volume (mL)	100	250	500	-	-	-
Citrate solution pH	-	-	-	8.4	9.4	10.4
Citrate concentration (M)	-	-	-	0.02	-	0.10
Reaction time (min)	-	-	-	30	-	120
Oil bath temperature (°C)	-	-	-	150	-	180

523 Table 3: Selected CQAs and acceptance criteria for the early characterization design

Critical Quality Attributes	Acceptance criteria
SERS signal intensity	> 15 000 counts
Relative standard deviation of SERS signal intensities	< 30%
Polydispersity index of AgNps suspension	< 0.3

524