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Designing Ultra-Small Nanostructured Lipid Carriers: Critical Process Parameters †

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Abstract: Nanoparticles (NPs) offer noteworthy advantages in the treatment of several diseases by prompting, among other benefits, site-specific delivery of drugs. Ultra-small nanostructured lipid carriers (usNLCs) are no exception. These correspond to a class of NPs composed of a blend of solid and liquid lipids, the latter usually in a higher proportion, which promote a less ordered solid lipid matrix, providing a higher drug loading capacity, drug release modulation, and improved stability in comparison with other lipid nanoparticles. Several manufacturing methods have been described for obtaining usNLCs. However, an in-depth process understanding is mandatory for a comprehensive knowledge allowing NPs property control. In the present work, the hot high-pressure homogenization (HPH) method, characterized by an easy scaling-up, simplicity, and ease of handling, is used to develop highly concentrated, small-sized NLCs. Critical process parameters (CPPs) and critical material attributes (CMAs) are assessed to address the reproducibility of the manufacturing process, consistency among batches, long-term stability of the formulation, drug loading capacity and drug release. In order to acquire an enhanced understanding of this method, a multivariate analysis is herein applied to inspect how the physicochemical properties of the usNLC are influenced by the variation of CPPs/CMAs. CPPs include HPH-time, HPH-pressure, while CMAs, such as lipid content, are also taken into consideration. The results show that a high lipid content (15% *w*/*w*), with an intermediate pressure and a short time in HPH seem to be the crucial parameters for obtaining both a small particle size (<100 nm) and a narrow size distribution (polydispersity index <0.2) in usNLC prepared by the hot-HPH method, without affecting zeta potential (>|30| mV).

Keywords: nanoparticles; lipid nanoparticles; critical process parameters; critical material attributes; ultra-small nanostructured lipid carriers

1. Introduction

Pharmaceutical development is an intensive and complex process. A good understanding of all stages is crucial to ensure the required quality of the final product. Thus, following the Quality by Design (QbD) concept makes it easier to understand the relationship between material attibutes and process parameters [1–3]. QbD is a systematic approach to development, based on prior knowledge and quality risk management of the formulation components and the production method, ensuring

the final product quality. In this way, QbD has been applied in the development of nanoparticles (NPs), aiming at simplifying and save costs in the manufacturing process by implementing quality specifications of the final product, as part of an overall control strategy.

Ultra-small nanostructured lipid carriers (usNLCs) gather particular colloidal features, which stand them as excellent candidates for drug delivery. In fact, they present several advantages, including (i) the use of physiological, biocompatible and biodegradable lipids, (ii) higher encapsulation efficiency, drug loading and stability, (iii) controlled drug release, (iv) incorporation of lipophilic and hydrophilic molecules [4]. Several methods (temperature—or organic solvent-based) have been described in literature for the production of usNLCs, including the hot or cold high pressure homogenization (HPH), melt dispersion, solvent emulsification-evaporation, hydrophobic ion paring, double emulsion, among others. However, only the HPH is established for large-scale production, with additional cost-effective advantages [5–8].

The understanding of the relationship between the critical process parameters (CPPs) and critical material attributes (CMAs) and the respective impact in the usNLC critical quality attributes (CQAs) is crucial for an increased performance. In particular, the correlation between the HPH processing time and pressure (as CPPs), and the lipid content (as CMA) are herein addressed. The influence of these variables on physicochemical parameters, such as particle size, polydispersity index and zeta potential were analyzed in order to obtain usNLCs with quality in terms of efficiency and safety for cancer therapy.

2. Experiments Materials

Polysorbate 80 (Tween® 80) was provided by Sigma. Capryol™ PGMC (propylene glycol monocaprylate-type I) and Precirol® ATO 5 were kindly offered by Gattefossé (Gennevilliers, France). Lipoid S 75® (soy phospholipid) was provided by Lipoid GmbH (Ludwigshafen am Rhein, Germany). Ultrapure water (HPLC grade, 18.2 MΩ was prepared by means of a Milli-Q water apparatus (Millipore®, Milford, MA, USA) and filtered through a 0.22 μm nylon filter before use.

2.1. Optimization of the Production Method of NLC

The usNLC were produced by hot high-pressure homogenization (hot-HPH). Modifications in terms of lipid content, pressure, and processing time in hot-HPH were evaluated to obtain the optimal conditions, according to a 3^3 full factorial design (see Table 1). Briefly, the lipid phase composed of a (25:75, % *w*/*w*) mixture of solid (Precirol® ATO 5) and liquid lipid (Capryol™ PGMC) and surfactant (1% *w*/*w*, Lipoid® S75) was prepared and heated up to 65 °C. In parallel, the aqueous surfactant phase containing Tween® 80 (5% *w*/*w*) was prepared and heated up to 65 °C before the addition to the lipid phase. The mixture was then homogenized using an Ultra-Turrax X 10/25 (Ystral GmBh, Dottingen, Germany) at 24,000 rpm for 1 min. The formed pre-emulsion was further processed by HPH (Emulsiflex C-3, Avestin, Mannheim, Germany), and the resulting dispersion was immediately cooled down to 4 °C. All samples were produced, at least, in triplicate.

Table 1. Design layout of different formulations.

Independent Variables	**Levels**		
	−1	**0**	**+1**
Lipid content (% *w*/*w*)	10	12.5	15
HPH time (min)	2.5	5	7.5
HPH pressure (bar)	500	1000	1500
Dependent variables	Particle size (PS)		
	Polydispersity index (PI)		
	Zeta potential (ZP)		

The physicochemical characterization of the usNLCs was performed in terms of particle size (PS), particle distribution (PI) and zeta potential (ZP) by dynamic and eletrophoretic light scattering,

using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25 °C. usNLC formulations were diluted with ultrapurified water (1:100) to generate an appropriate scattering intensity.

2.2. Multivariate Analyses

A multivariate analysis is herein applied to inspect how the physicochemical properties of the usNLCs were influenced by the variation of CPPs and CMAs. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed, resorting to JMP Pro 15.0.0 (SAS Institute Inc., Cary, NC, USA). Both methods require a spatial description of the usNLC formulations as points in Euclidean space. HCA and PCA are tools to explore hidden patterns, similarities, and differences among samples, where the relationships within the data are not readily visible. HCA and PCA were applied after standard normalization to evaluate the quantitative and qualitative effects of the CPPs and CMAs on PS, PI, and ZP. HCA and PCA were performed on the data set comprehending 27 different formulations. A total of 3 defined predictors were considered corresponding to the formulation lipid content and the processing time and pressure in HPH during the usNLCs preparation. In HCA, the distance between clusters was calculated using Ward's minimum variance method, whereas PCA models were determined using the Row-wise estimation method and the correlation matrix.

3. Results

Optimization and Production of Unloaded usNLCs

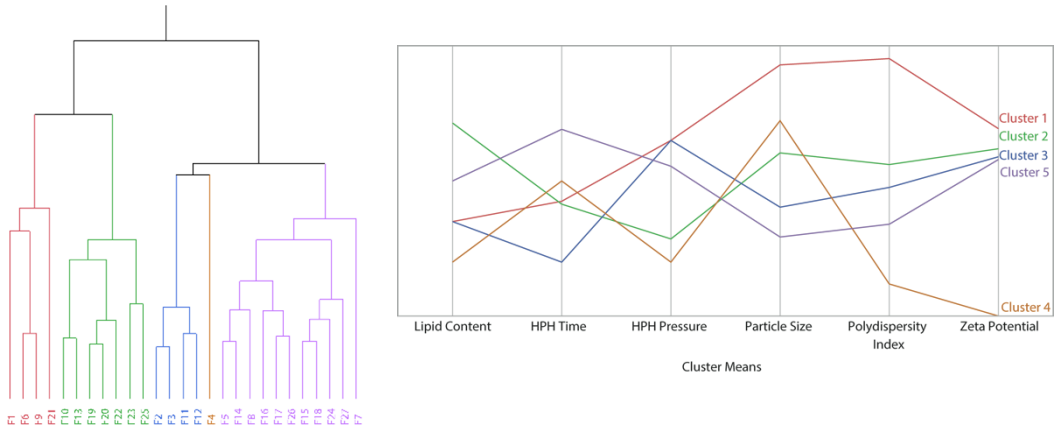
Identifying the critical parameters, in terms of composition and processing, and inspecting how they will affect the final dispersion is a major step in the development of nanocarriers. Multivariate analysis is a practical approach to evaluate the influence of independent variables (CPPs and CMAs, HPH pressure and time, and lipid content, respectively) on the dependent variables (CQAs). These CQAs, including PS, PI, and ZP, ultimately determine the physicochemical properties of the usNLCs. In fact, these properties can influence their drug loading capacity, drug release behavior, stability in aqueous and biological media, biocorona formation tendency as well as other in vitro/in vivo parameters. Selecting the most suitable components and their concentrations requires a careful planning and optimization to achieve the desired outcome. The ideal PS, PI, and ZP of the usNLCs were observed to be as low as possible PS and PI, and higher than |30| mV for zeta potential, along with the highest lipid content (Table 2). The composition trends can be monitored by combining (i) HCA taking advantage of the hierarchical distribution (Figure 1); (ii) PCA, with a biplot representation of the first two principal components (Figure 2) and (iii) a full factorial design, represented by contour plots (Figure 3).

Table 2. Three-level, three-variable, 3^3, full factorial design for the optimization of the production method of usNLCs. The results are expressed as mean ± standard deviation (SD). Formulations are highlighted in color, according to the clusters defined by HCA. Key: F: Formulation; LC: Lipid content; HPH: high pressure homogeneization; PS: particle size; ZP: zeta potential; PI: polydispersity index.

F	LC (%)	HPH Time (min)	HPH Pressure (bar)	PS	PI	ZP
1	10	2.5	500	201 ± 0.4	0.414	−34.3 ± 0.4
2	10	2.5	1000	112 ± 0.5	0.253	−36.0 ± 0.5
3	10	2.5	1500	113 ± 0.4	0.254	−36.0 ± 0.4
4	10	5	500	153 ± 1	0.161	−43 ± 1
5	10	5	1000	110 ± 1	0.245	−38 ± 1
6	10	5	1500	171 ± 2	0.333	−35 ± 2
7	10	7.5	500	120 ± 1	0.160	−29 ± 1
8	10	7.5	1000	106 ± 1	0.205	−34 ± 1
9	10	7.5	1500	157 ± 1	0.386	−35 ± 1
10	12.5	2.5	500	116 ± 2	0.256	−38 ± 2

11	12.5	2.5	1000	119 ± 1	0.260	−35 ± 1
12	12.5	2.5	1500	115.7 ± 0.4	0.260	−32.0 ± 0.4
13	12.5	5	500	137 ± 1	0.266	−37 ± 1
14	12.5	5	1000	97 ± 2	0.246	−37 ± 2
15	12.5	5	1500	100 ± 1	0.247	−35 ± 2
16	12.5	7.5	500	100.5 ± 0.6	0.251	−37 ± 1
17	12.5	7.5	1000	98 ± 1	0.253	−33 ± 1
18	12.5	7.5	1500	96 ± 2	0.212	−34 ± 2
19	15	2.5	500	125 ± 1	0.264	−32 ± 2
20	15	2.5	1000	138 ± 1	0.257	−32 ± 1
21	15	2.5	1500	180.4 ± 0.3	0.406	−28.0 ± 0.3
22	15	5	500	138 ± 2	0.230	−34 ± 2
23	15	5	1000	170 ± 1	0.331	−32 ± 1
24	15	5	1500	102 ± 1	0.192	−32 ± 1
25	15	7.5	500	150 ± 1	0.352	−36 ± 1
26	15	7.5	1000	100.1 ± 0.2	0.206	−34.5 ± 0.2
27	15	7.5	1500	92 ± 2	0.203	−38 ± 2

Figure 1B provides an overview of the data structure, identifying the groups of usNLCs sharing similar properties. Thus, it is possible to evaluate the usNLCs considering different formulations and process parameters. Five clusters are identified. Cluster 1 (red) represents usNLCs with high (>150 nm) size and a PI > 0.250; cluster 2 (green) represents the high lipid content usNLC with high PS > 155 nm; cluster 3 (blue) and 5 (purple) clusters show small usNLCs (≤100–140 nm) with a narrow distribution <0.260, but produced with different HPH processing times (5 and 7.5 min); finally, cluster 4 (brown) displays the usNLCs with a a high PS (>150 nm) but with a narrow distribution (0.160). The analysis shows that it is possible to increase the lipid content while obtaining a small sized-formulation. During production, it is important to describe the relationship between HPH time and pressure. Figure 2B shows an easier interpretation for each cluster, confirming the similarity profile between formulations and the mean value to each corresponding variable in the respective cluster.



(**A**) (**B**)

Figure 1. (**A**) Hierarchical cluster analysis (HCA) of an experimental conditions taking into consideration their similarity in the expression of CQAs. (**B**) Cluster means representation.

Figure 2 presents the contribution of each independent and dependent variable, representing the 27 usNLC formulations. CPPs, as HPH pressure and HPH time, and CMAs, as lipid content, are explained by the first two principal components (PC), PC1, and PC2, which suggest the variables responsible for the discrimination of the formulations. The representation of data on the first two PCs is an easy and straightforward way to visualize and understand the relation between composition,

process parameters and CQAs, discriminating the usNLCs formulations. The biplot allows visualizing the scores and loadings representing the coefficients of usNLCs, CPPs, CMAs and CQAs on the two principal components. The interpretation of the relative positioning of formulations on both direction and length of the loadings is also important. PC1, the first component, retains information of the HPH time, while PC2, the second component, is associated to HPH pressure and lipid content. PS and PI contribute positively to PC1 and ZP to PC2. Therefore, HPH time opposes PS and PdI. This means a higher HPH time leads to a lower PS and narrow size distribution. HPH pressure and the lipid content are in the PC2 opposite direction, meaning that both parameters influence ZP. However, a higher amount of lipid content and higher HPH pressures lead to smaller ZP values. The effect of HPH pressure should be carefully inspected. High homogenization pressure might result in a decrease of the absolute value of ZP. Indeed, when the number of particles with high kinetic energy is increased, particle collision is favored, which may result in usNLC with lower stability. In spite of all these trends, it should be noted that the ZP values remained above |30| mV, ensuring formulation stability.

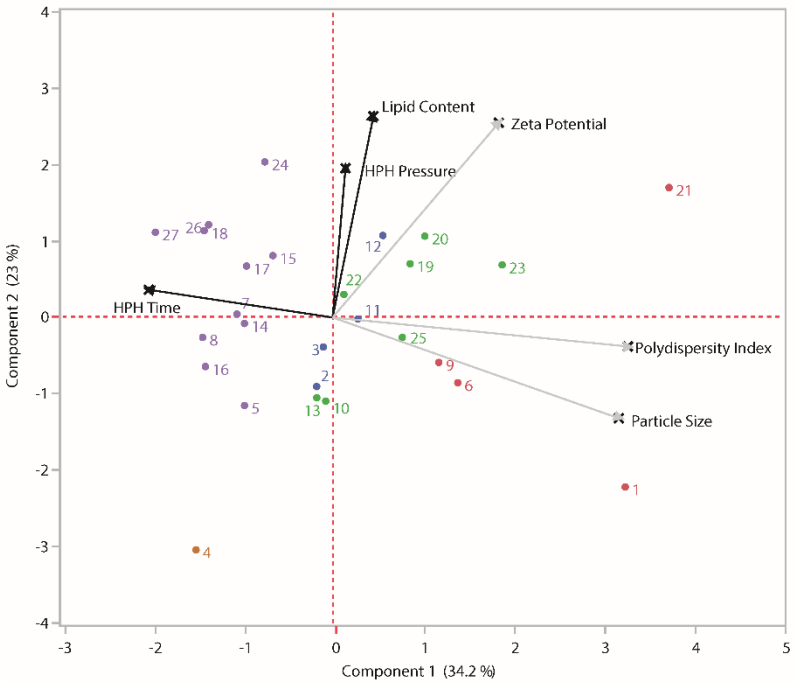
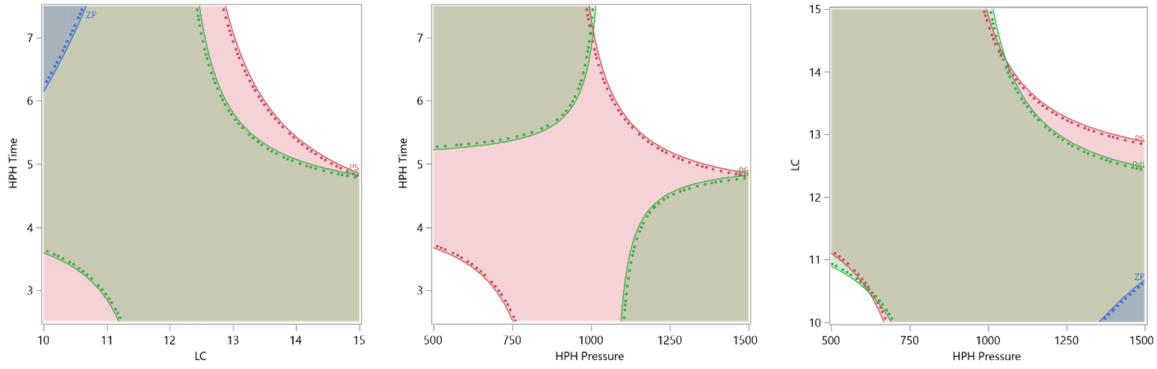


Figure 2. Principal component analysis (PCA) plotted along the first two axes, with the representation of the 27 usNLCs formulations and the 3 variables corresponding to usNLCs components and CQAs, on the first two components, recovering 57.2% of variance.

After understanding the similirities and the patterns among formulations, a 3^3 full factorial design was performed, now aiming at weighing the effect of the process parameters and the lipid content on the colloidal properties (PS, PI and ZP) of the usNLCs. Figure 3 exhibits the operable region for the optimized formulations. The model predicts that longer times in HPH (7.5 min), high lipid content (15% *w*/*w*) and high pressures in HPH (ranging from 1000 and 1500 bar) result in usNLCs of sizes below 100 nm, narrow size distributions and ZP > |30| mV. Again, ZP is a parameter which does not seem to be influenced by these variables (see Figure 3).



(**A**) (**B**) (**C**)

Figure 3. Particle size, polydispersity index and zeta potential contour plots for (**A**) HPH time vs. LC, (**B**) HPH time vs. HPH Pressure and (**C**) LC vs. HPH Pressure. The operable area is highlighted (white). Each missing factor was set to the maximum level, in order to optimize the CQAs.

4. Discussion

In a previous study [9,10], it was possible to identify some key factors and understand which of them influence the mean particle size. These works addressed CMAs, such as lipid concentration, the ratio between liquid and solid lipids, surfactant type, concentration, and CPPs, such as Ultra-Turrax time, HPH pressure, and batch volume. Aiming now at increasing the lipid content, from 7.5 to 15% *w*/*w*, and keeping particle size below 100 nm, it is important to evaluate the influence of the usNLC production method and, consequently, the impact of the increase of lipid content upon usNLC properties. QbD approach helped to understand the behavior between CPPs and CMAs in the hot-HPH method and to increase the lipid content when compared to previous works [10–13]. It seems clear that usNLCs containing a higher lipid content render larger particle sizes. However, with process parameters adjusted, longer HPH time, and high pressures, it is possible to obtain particle sizes around 100 nm even with a higher lipid content. A remarkable correlation among the tested parameters and the mean particle diameter was found. A high HPH pressure along with extended HPH time results in smaller usNLC for the tested set-up. Furthermore, narrow PI are associated with high pressures, 1000 and 1500 bar, irrespective of the time. Attempting a translation to cancer drug delivery, usNLC with these properties are expected to hold an enhanced permeability and retention (EPR) effect with a preferential accumulation in the tumor tissues. Also, particle size stands as a critical parameter to intravenous administration and biological barriers permeation. However, future studies must be carried out to explore the therapeutic potential of usNLCs in cancer.

5. Conclusions

The optimization method based on QbD was successfully implemented, as a surrogate to the conventional trial-and-error approach. Multivariate analyses were applied to infer about the critical parameters that influence the physicochemical characteristics, such as PS, PI, and ZP, on usNLC formulated by the hot-HPH method. Understanding the parameters that should be used in the hot-HPH method for usNLC represents a useful approach for subsequent optimization and characterization steps. The optimal formulation was obtained in terms of size and polydispersity with a high lipid content (15% *w*/*w*), a long time in HPH (7.5 min), and a medium HPH pressure (1000 bar). In conclusion, this work provides crucial information on the process parameters and critical materials attributes for usNLC production directed to cancer therapy.

Author Contributions: M.M. performed the experiments and analyzed the data; J.J., A.P. and C.V. contributed reagents/materials/analysis tools; M.M., J.B., J.S., A.P. and C.V. contributed to the writing and editing of the paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

usNLC:	ultra-small nanostructured lipid carriers
NPs:	Nanoparticles
QbD:	Quality by Design
HPH:	high pressure homogeneization
CPP:	Critical process parameters
CMAs:	Critical material attributes
CQAs:	Critical quality attributes
LC:	lipid content
PS:	particle size
PI:	polydispersity index
ZP:	zeta potential
F:	Formulation

References

1. Patel, G.M.; Shelat, P.K.; Lalwani, A.N. QbD based development of proliposome of lopinavir for improved oral bioavailability. *PHASCI* **2016**, doi:10.1016/j.ejps.2016.08.057.
2. Politis, S.; Colombo, P.; Colombo, G.; M Rekkas, D. Design of experiments (DoE) in pharmaceutical development. *Drug Dev. Ind. Pharm.* **2017**, *43*, 889–901, doi:10.1080/03639045.2017.1291672.
3. Cunha, S.; Costa, C.P.; Moreira, J.N.; Lobo, J.M.S.; Silva, A.C. Using the quality by design (QbD) approach to optimize formulations of lipid nanoparticles and nanoemulsions: A review. *Nanomed. Nanotechnol. Biol. Med.* **2020**, *28*, 102206.
4. Tamjidi, F.; Shahedi, M.; Varshosaz, J.; Nasirpour, A. Nanostructured lipid carriers (NLC): A potential delivery system for bioactive food molecules. *Innov. Food Sci. Emerg. Technol.* **2013**, *19*, 29–43.
5. Martínez-Monteagudo, S.I.; Yan, B.; Balasubramaniam, V.M. Engineering Process Characterization of High-Pressure Homogenization—From Laboratory to Industrial Scale. *Food Eng. Rev.* **2017**, *9*, 143–169.
6. Dingler, A.; Gohla, S. Production of solid lipid nanoparticles (SLN): Scaling up feasibilities. *J. Microencapsul.* **2002**, *19*, 11–16.
7. Gupta, S.N.R.C.N. *Advances in Nanomedicine for the Delivery of Therapeutic Nucleic Acids*; Elsevier: Amsterdam, The Netherlands, 2017; ISBN 9780128097175.
8. Thassu, D.; Deleers, M.; Pathak, Y.V.; Pathak, Y. *Nanoparticulate Drug Delivery Systems*; Drugs and the Pharmaceutical Sciences; Taylor & Francis: Abingdon, UK, 2007; ISBN 9780849390739.
9. Mendes, M.; Soares, H.T.; Arnaut, L.G.; Sousa, J.J.; Pais, A.A.C.C.; Vitorino, C. Can lipid nanoparticles improve intestinal absorption? *Int. J. Pharm.* **2016**, *515*, 69–83.
10. Barone, A.; Mendes, M.; Cabral, C.; Mare, R.; Paolino, D.; Vitorino, C. Hybrid Nanostructured Films for Topical Administration of Simvastatin as Coadjuvant Treatment of Melanoma. *J. Pharm. Sci.* **2019**, *108*, 3396–3407.
11. Mendes, M.; Soares, H.T.; Arnaut, L.G.; Sousa, J.J.; Pais, A.; Vitorino, C. Can lipid nanoparticles improve intestinal absorption? *Int. J. Pharm.* **2016**, *515*, 69–83.
12. Mendes, M.; Basso, J.; Silva, J.; Cova, T.; Sousa, J.; Pais, A.; Vitorino, C. Biomimeting ultra-small lipid nanoconstructs for glioblastoma treatment: A computationally guided experimental approach. *Int. J. Pharm.* **2020**, *587*, 119661, doi:10.1016/j.ijpharm.2020.119661.
13. Basso, J.; Mendes, M.; Silva, J.; Sereno, J.; Cova, T.; Oliveira, R.; Fortuna, A.; Castelo-Branco, M.; Falcão, A.; Sousa, J.; et al. Peptide-lipid nanoconstructs act site-specifically towards glioblastoma growth impairment. *Eur. J. Pharm. Biopharm.* **2020**, *155*, 177–189, doi:10.1016/j.ejpb.2020.08.015.