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Optimization of Protein Precipitation for High-Loading Drug Delivery Systems for Immunotherapeutics [†]

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Abstract: Cancer is the second leading cause of death in the world and is often untreatable. Protein-based therapeutics, such as immunotherapeutics, show promising results in the fight against cancer, resulting in their market share increasing every year. Unfortunately, most protein-based therapeutics suffer from fast degradation in the blood, making effective treatment expensive, causing more off-target effects (due to the high doses necessary), and often require repeated injections to stay within the correct therapeutic range. Encapsulation of these proteins inside nanocarriers are prompted to overcome these problems by enhancing targeted drug delivery and, thus, leading to a less frequent administration and lower required dose. However, most current protein encapsulation methods show very low loading capacities (LC). This leads to even more expensive treatment and might pose further risk for the patient caused by systemic toxicity against high concentrations of carrier material. We investigated and optimized protein nanoprecipitation as a method to obtain a high protein LC and encapsulation efficiency (EE) inside poly(lactic-co-glycolic acid) (PLGA) nanoparticles via a simple two-step process. In this work we used model proteins to investigate the influence of various parameters such as precipitation solvent, addition speed and protein concentration on the protein activity. Our work is a critical step towards high-loading encapsulation of immunotherapeutics.

Keywords: nanoprecipitation; protein desolvation; protein therapeutics; nanoparticles; PLGA nanoparticles; drug delivery; nanoencapsulation; immunotherapeutics

1. Introduction

Cancer is the second leading cause of death in the world and is often untreatable [1,2]. However, protein therapeutics have revolutionized the oncology field, showing promising results in the fight against cancer [3]. Unfortunately, while performing well *in vitro*, protein-based active pharmaceutical ingredients (APIs) are often rapidly cleared from the blood [4] and are less effective *in vivo*. To overcome these problems, high doses are administered in short intervals, thus increasing costs and invasiveness of the procedure. Encapsulation of these protein-based APIs inside nanoparticles could prevent protein degradation, promote passive or active targeting and reduce side effects [4,5]. However, the nanoparticles should remain below 200 nm in size, in order to prevent quick opsonization by the reticuloendothelial system, prevent particle filtration by the spleen and still make use of the enhanced permeability and retention effect [6,7]. Moreover, most current protein encapsulation methods show very low loading capacities (LC) or remaining protein activity after

encapsulation. In this paper, protein nanoprecipitation is investigated as a method for encapsulation of high concentrations of protein inside poly(lactic-co-glycolic acid) (PLGA) nanoparticles.

Nanoprecipitation, also known as desolvation, was first described by Fessi et al. [8] in 1989 and involves two miscible solvents, one of which is a good solvent for the polymer and API (e.g., acetone), while the other is a bad solvent (e.g., water). When this good solvent containing polymer and API is added to a large volume of the bad solvent, API encapsulated nanoparticles are formed (for a detailed review of the mechanism, see [9]). This method works well for hydrophobic drugs that can be dissolved together with the polymer. However, most proteins are not directly soluble or lose their activity in these organic solvents, making this original method unsuitable for protein encapsulation. Recently, Morales-Cruz et al. [10] introduced a modified, two-step nanoprecipitation method for encapsulation of proteins inside PLGA nanoparticles. In this case, the protein was precipitated into protein nanoparticles, mixed with PLGA, and then precipitated, creating protein-loaded PLGA nanoparticles. However, this method only encapsulated smaller proteins (12–21 kDa) and it remains uncertain if this method can be used for encapsulation of larger immunotherapeutics (~ 150 kDa). In this work, two model proteins, bovine serum albumin (BSA) (66.5 kDa) and amylase (51–54 kDa), which are two times larger than previously reported, were precipitated by the non-solvent (NS) acetonitrile (ACN) and encapsulated inside PLGA nanoparticles. We have tested various parameters that may affect the protein and polymer precipitation and demonstrated that the activity of amylase is preserved during the process.

2. Materials and Methods

2.1. Chemicals

Poly(lactic-co-glycolic acid), PLGA 5002 (50:50) and PLGA 5002A (50:50) were kindly provided as a gift by Corbion. All solvents were purchased from VWR. Bovine serum albumin (BSA)(K35-011) was purchased from PAA laboratories. Amylase (10044725) was purchased from MP biomedical.

2.2. Protein Precipitation

The protocol was adapted from [10] with few modifications. 200 μ l of protein of various concentrations, amylase or BSA, was precipitated by dropwise (0.5 mL/min) addition of NS (ACN, ethanol, acetone) at various NS:water ratios while stirring (500 rpm). Used concentrations, solvents and ratios will be further indicated in the text. After addition the samples were incubated for 5 min at room temperature and their size and distribution characterized using DLS.

2.3. PLGA Nanoprecipitation

The protocol was adapted from [10] with few modifications. 100 μ l of PLGA (5002 or 5002A) in ACN:water (5.5:1 ratio) at various concentrations (3.3, 6.8, 10.2, 13.4 mg/mL, final concentration), was slowly added to the precipitated protein mixture (described above) under stirring (500 rpm). Once PLGA was mixed, the samples were immediately precipitated. The precipitation was done by dropwise addition at 0.1 mL/min of 1 mL of PLGA/protein mixture to 9, 19 or 39 mL of water containing 10 mg/mL of F127 or F68 under stirring (700 rpm). Blank nanoparticles without protein were also created. The PLGA concentrations and water volume are further indicated in the text.

2.4. PLGA Solubility

The solubility of PLGA (5002, 5002A) in ACN/water mixtures was tested by dropwise addition of 10 μ l of water to 19 mg of PLGA in ACN for various concentrations, till visible precipitation was observed. The maximum water percentage was recorded as the last addition before precipitation.

2.5. Dynamic Light Scattering

The protein and PLGA nanoparticles were analyzed by dynamic light scattering (DLS) (NanoPhox, Sympatec) at 25 °C. Protein nanoparticles were measured directly as a suspension in

their NS:water mixture and PLGA particles were measured as suspension in water with surfactant. Samples were diluted if the observed kilocounts per second (KCPS) succeeded 500 KCPS. Further data analysis was performed using Python 3.0.

2.6. Encapsulation Efficiency and Protein Activity

The encapsulation efficiency (EE) was measured indirectly by spinning down the protein particles for 20 min at 22,000 RCF (Centurion Scientific Benchtop Centrifuge), and measuring the protein concentration in the supernatant, following standard microBCA assay (ThermoFisher). Absorbance was measured at 562 nm (microplate reader, SpectraMax). The EE was calculated using the following equation:

$$EE (\%) = \frac{\text{theoretical total amount of protein} - \text{measured protein in supernatant}}{\text{theoretical total amount of protein}} \times 100$$

For activity measurements, 2 mg/mL of amylase was precipitated as described above. After precipitation, 1 mL of precipitated protein was directly added to 14 mL of PBS. The protein was then incubated at 4 °C for at least 2 h to let the protein fully redissolve. 2 mg/mL stock amylase was diluted (100×) in PBS and the activity of both samples was measured following standard amylase activity colorimetric assay kit (BioVision, K711) protocol at 405 nm (microplate reader, SpectraMax). The measured activity of redissolved protein was then normalized against the activity of stock amylase.

3. Results and Discussion

Protein nanoencapsulation by nanoprecipitation requires two steps: first, nanoprecipitation of protein, second, nanoencapsulation of this precipitated protein by PLGA during a second nanoprecipitation step. In order to preserve the activity of the protein throughout the steps and obtain nanoparticles below 200 nm with high EE, each step has to be carefully optimized. In the following sections we discuss factors affecting each process: protein nanoprecipitation, PLGA nanoprecipitation and encapsulation.

3.1. Protein Precipitation

Protein precipitation is the first step that may lead to the loss of protein activity. In order to get a better understanding of protein nanoprecipitation three different parameters were investigated: NS, addition speed, and initial protein concentration.

The NS in protein nanoprecipitation can have a big influence on protein particle size, polydispersity index (pdi) and stability. Ethanol is most commonly used as a NS in protein nanoprecipitation of BSA [11,12]. However, PLGA is needed in the second precipitation step and is only soluble in ACN or acetone. When comparing ACN and acetone for protein precipitation of lysozyme and alpha-chemotrypsin, Morales-cruz et al. [10] observed that ACN induced the least amount of non-soluble aggregates and retained the highest protein activity. To find the best precipitation conditions for proteins of interest, we have tested three water miscible solvents for BSA precipitation: acetone, ACN and ethanol.

As shown in Figure 1a, only precipitation with ACN lead to a homogeneous distribution of BSA nanoparticles below 200 nm. Furthermore, ACN lead to the highest particle count on DLS (+ 500 KCPS), indicating a large amount of protein particles. According to Von Storp et al. [13], the higher the dielectric constant of the non-solvent (ACN > ethanol > acetone), the smaller the formed protein particles should be. However, due to the broad distribution of BSA particles after ethanol and acetone precipitation, these findings could not be compared. Furthermore, even though ACN is not often used for protein precipitation in literature, it showed a homogeneous distribution and high concentration of protein particles, making it a good candidate for two step precipitation. ACN was therefore selected as solvent for all further experiments.

One of the possible explanations of the broad particle size distributions observed in Figure 1a is a too fast addition speed. Paik et al. [12] also observed larger BSA protein particles at fast addition of ethanol, and showed that slow, controlled addition to BSA leads to robust size control. They

suggested that slower addition stabilizes particle formation by allowing for more equilibrium time. As shown in Figure 1b, instant addition of ACN to BSA solution lead to smaller particles, but the resulting particle size distribution was larger. Furthermore, some larger aggregates were observed in the same solution (~200 nm). On the other hand, slow and controlled addition (controlled by a syringe pump) of ACN lead to repeatable, larger, and more homogeneously distributed particles (see Figure 1c). Therefore, slow addition of ACN was used to further investigate the effect of initial BSA concentration on protein particle size and distribution.

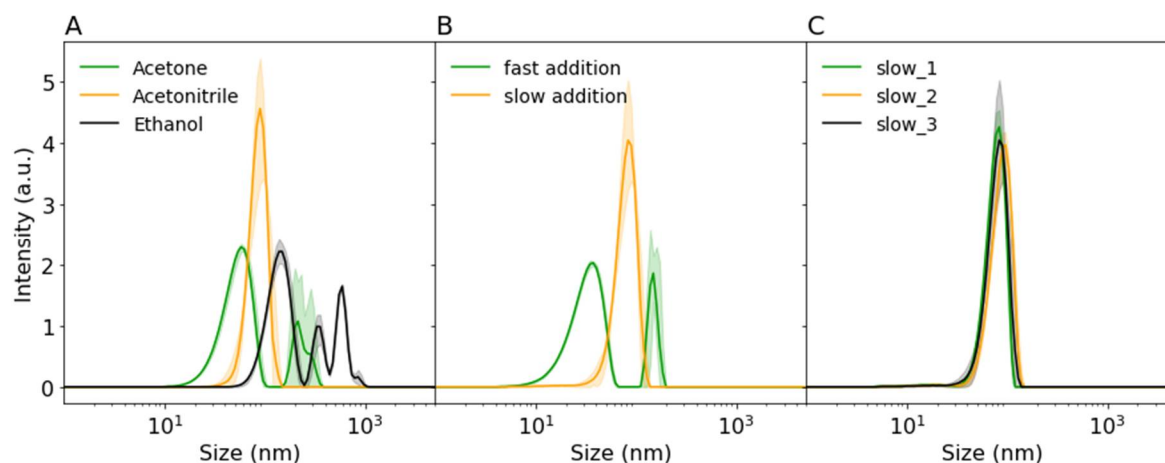


Figure 1. DLS intensity distributions of (a) precipitated 12.5 mg/mL BSA by various non-solvents (1:4 water:acetonitrile (ACN)), (b) the influence of addition speed of ACN (fast = instant pipetting, slow = 0.5 mL/min) on nanoprecipitation of 25 mg/mL BSA, (c) repeatability of precipitation of 25 mg/mL BSA by slow addition (0.5 mL/min) of ACN. Shaded areas indicate standard deviation.

Another factor that may influence the size of nanoprecipitated proteins is their concentration [11,14]. Only minor differences in protein size were found by Tarhini et al., with the lowest concentration leading to the smallest nanoparticles. Rahimnejad et al. [14] showed that initially the protein particle size decreased with increasing concentration, but beyond a certain point the particle size remained the same. Here, BSA was precipitated in a range between 10 and 25 mg/mL at 5.5:1 ACN:water ratio. As shown in Figure 2a and Table 1, in our case the size of the BSA protein particles increases with increasing protein concentration, from 35.2 ± 0.2 nm to 67.0 ± 4.1 nm for 10 mg/mL and 25 mg/mL, respectively. The increase appears to be linear within this given range.

As a model protein for activity measurement, amylase was precipitated. Due to lower solubility, a lower initial concentration (2 mg/mL) of amylase was used. Interestingly, the particle size of nanoprecipitated amylase was a lot larger than for BSA, 133.7 ± 4.7 nm (see Figure 2a and Table 1). This suggests that the precipitation process may be influenced by certain unique protein properties (hydrophobicity, molecular weight or charge) and optimized precipitation parameters for one protein might not lead to similar results for others. Therefore, the initial protein concentration might be used to alter the protein particle size of specific proteins, but is not a universal method to control particle size.

There are many publications where precipitated and cross-linked protein nanoparticles are used for encapsulation of other APIs [11,13,15–17]. However, in order to use this method for protein encapsulation of protein immunotherapeutics, the proteins should be able to redissolve in aqueous solution and retain their activity. Morales-Cruz et al. [10] showed that lysozyme and alpha-chymotrypsin remain active after protein nanoprecipitation using ACN. To evaluate the influence of the nanoprecipitation on activity of large proteins, amylase nanoparticles were redissolved in PBS and their activity quantified. As seen in Figure 2c, redissolved amylase retained 101.1 ± 5.4 % of activity compared to stock solution. This seems to suggest that nanoprecipitation of amylase does not lead to irreversible denaturation.

In conclusion, our results suggest that the first protein nanoprecipitation step is not detrimental to the protein structure and might be applicable for a variety of proteins.

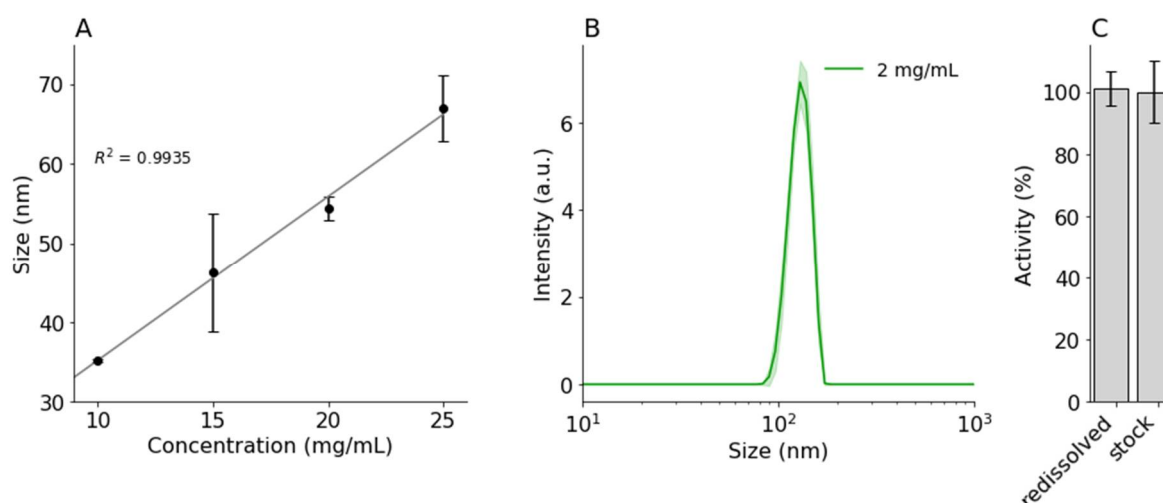


Figure 2. DLS intensity distributions of (a) nanoprecipitated BSA (10,15,20,25 mg/mL) and (b) amylase (2 mg/mL) with ACN (5.5:1) (0.1 mL/min). (c) Normalized activity of redissolved nanoprecipitated amylase in PBS, compared to amylase stock. Error bars and shaded areas indicate standard deviation.

Table 1. Overview of particle size (z-average) and polydispersity index (pdi) of acetonitrile precipitated BSA and amylase at various concentrations.

Protein	Concentration (mg/mL)	z-Average (nm)	pdi
BSA	10	35.2 ± 0.2	0.18 ± 0.035
	15	46.2 ± 7.4	0.13 ± 0.055
	20	54.3 ± 1.5	0.15 ± 0.019
	25	67.0 ± 4.1	0.13 ± 0.032
amylase	2	133.7 ± 4.2	0.013 ± 0.007

3.2. PLGA Precipitation

The second step of protein nanoencapsulation is the encapsulation of the obtained protein nanoparticle inside PLGA. For that, PLGA should be dissolved into the protein suspension and the whole solution precipitated out in a NS (water) for PLGA. One of the most critical parameters affecting this nanoprecipitation step is the polymer solubility in the solvent mixture. That is, if PLGA already precipitates out of solution upon addition to the protein mixture, the protein will not be encapsulated when the PLGA/protein mixture is precipitated in water. In our preliminary experiments, the proteins were precipitated by ACN addition to a final 4:1 ACN:water ratio, since this ratio lead to sufficient protein precipitation. However, addition of PLGA in any concentration to this 4:1 ACN:water ratio, lead to immediate nanoprecipitation of PLGA. The water concentration was too high to keep PLGA dissolved. As shown in Table 2, PLGA nanoprecipitates out of ACN when it contains a water concentration between 16 and 18%. Therefore, the initial 4:1 ratio was raised to 5.5:1 and used to investigate other factors that may influence PLGA nanoprecipitation.

Table 2. Highest acceptable water percentage in acetonitrile/water mixtures before PLGA precipitates out of solution. Increments of 10 ul of water were added to 19 mg of PLGA (5002, 5002A) in acetonitrile at various concentrations, till visible precipitation occurred.

PLGA	Concentration (mg/mL)	ACN (µL)	H ₂ O (µL)	Max H ₂ O (%)
5002	190	100	20	16.67
	95	200	40	16.67

	47.5	400	90	18.37
5002A	190	100	20	16.67
	95	200	40	16.67
	47.5	400	90	18.37

The ratio of NS:water used for protein precipitation in our experiments was higher than previously reported. To validate the published observations on PLGA nanoprecipitation in our setup, the effects of PLGA concentration, surfactants, and organic:aqueous phase ratio were investigated, without the protein particle suspension. The surfactants in the water phase are supposed to stabilize the particles during the nucleation and growth process and are necessary in obtaining stable sub 200 nm particles [9]. In agreement with Lebouille et al., the PLGA particles aggregate into bigger microparticles upon addition to water if surfactants are not used (see Figure 3a). Another factor that may affect PLGA precipitation is the ratio of organic to aqueous phase. The organic phase needs to be precipitated in an excess of water, however larger excess will lead to a lower final concentration of particles, potentially requiring large volumes or up-concentration to be effective *in vivo*. In our case a decrease in water phase ratio from 1:39 to 1:9 did not notably affect particle size or size distribution (see Figure 3b). Therefore, in our setup a 1:9 ratio already provides enough excess volume for successful precipitation, leading to higher particle concentrations. Finally, we also tested the influence of PLGA concentration on particle size. As shown in Figure 3c, PLGA particle size decreases from 181.3 ± 0.34 nm to 80.5 ± 0.75 nm with a decreasing PLGA concentration from 13.4 mg/mL to 3.3 mg/mL respectively. Our results suggest that the final particle size could be controlled by adjusting the PLGA concentration. However, addition of protein particles inside this PLGA mixture might influence the precipitation outcome. Amylase encapsulation inside PLGA is discussed in the following section.

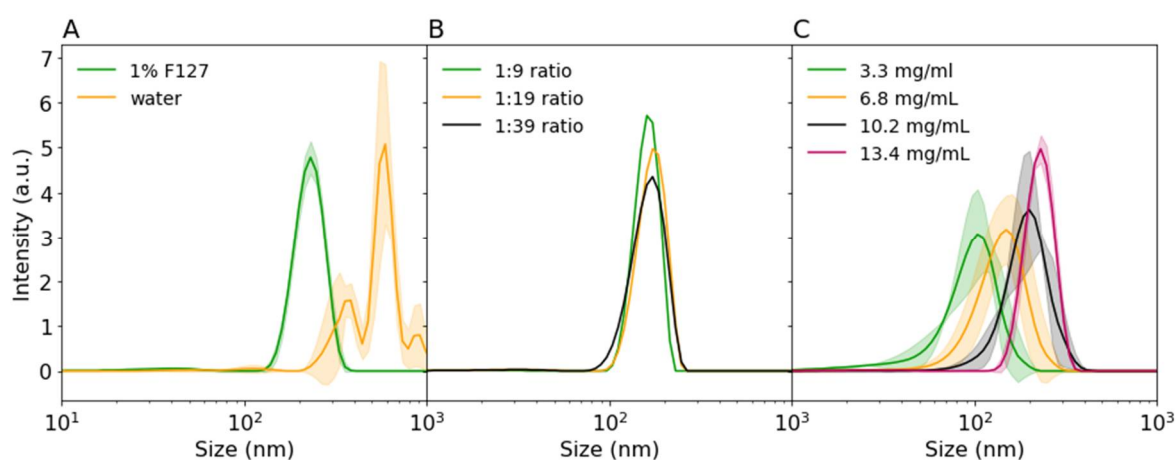


Figure 3. DLS intensity distributions of (a) precipitated 5002A PLGA in water of 1% F127 (1:9 ratio), (b) precipitated 5002 PLGA in different 1% F127 ACN:water ratios, (c) precipitated 5002A PLGA at various PLGA concentrations in 1% F127 (1:9 ratio). Shaded areas indicate standard deviation.

3.3. Encapsulation

Finally, for practical applications therapeutical proteins should be encapsulated for drug delivery and on-target release. As a proof of concept amylase was encapsulated inside PLGA nanoparticles using the optimized conditions discussed in previous sections. First, amylase was precipitated with ACN to form protein nanoparticles (133.7 ± 4.2 nm). Then 10 mg/mL PLGA (final concentration) was added to the suspension, and the whole solution was precipitated in 1% F68 solution. As shown in Figure 4a, 147.7 ± 4.3 nm nanoparticles were formed. In this case, only a small (~14 nm) increase in particle size was detected, suggesting that only a small polymer shell was formed. This may be beneficial for a faster protein release. On the other hand, as seen in Figure 4b,

only a small amount, 24 ± 11.2 %, of amylase was encapsulated or adsorbed onto the PLGA nanoparticles.

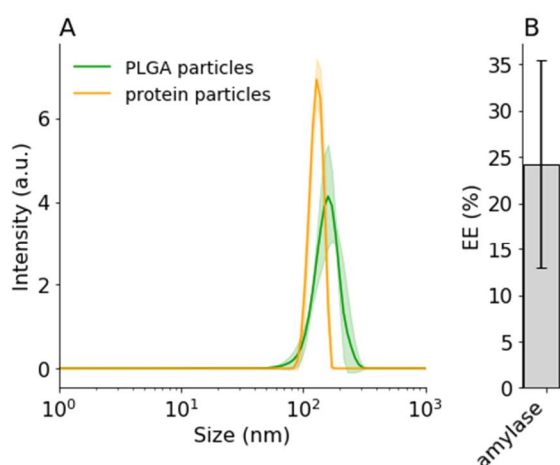


Figure 4. DLS intensity distribution of (a) precipitated amylase (5.5:1 ACN:water) and encapsulated amylase (5002A, 1:29, 1% F68) (b) Encapsulation efficiency of amylase. Error bars and shaded areas indicate standard deviation.

Morales et al. has also observed low EE (~30%) of alpha-chemotrysin [10]. They improved the EE by lowering the initial protein concentration, i.e., effectively decreasing the size of the protein nanoparticles. This effect of initial protein particle size on the EE will be investigated in our further experiments. Moreover, the influence of the PLGA polymer shell on the release properties of such nanoparticles will also be studied.

4. Conclusions

In this work we have studied the influence of various process parameters on nanoprecipitation and encapsulation of proteins. We have investigated the influence of several solvents and addition speed on the nanoprecipitation of proteins and showed that slow addition (0.1 mL/min) of acetonitrile is a reproducible method to obtain small (<200 nm) protein nanoparticles. We have also found the optimum non-solvent:water ratio, 5.5:1, needed for effective two-step nanoprecipitation with PLGA. Finally, we have successfully precipitated amylase without losing its activity and encapsulated amylase nanoparticles in PLGA. Overall, our work validated the feasibility of larger protein precipitation that is an important step toward nanoprecipitation of immunotherapeutics.

Author Contributions L.C.N. conceived, designed and performed the experiments; L.C.N. analysed the data; L.C.N. and A.S. wrote the paper; M.B. and A.S. supervised the work and contributed insights and discussions to the theory and manuscript.

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

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