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Wet spherical agglomeration of proteins as a new method to prepare parenteral fast soluble dosage forms

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Summary

Using a wet spherical agglomeration technique it was possible to form agglomerates from a powder containing proteins by suspending it in an organic liquid and adding a small amount of an aqueous liquid with a pH that guarantees good stability of the protein. Regarding the size distribution of the agglomerates an optimal amount of aqueous bridging liquid could be defined. Using bovine serum albumin (BSA) as a low-cost model protein, the influence of various parameters on the agglomeration process was evaluated. The disintegration time could be influenced by the type and the amount of added excipient. The optimal amount of bridging liquid needed could be predicted at each protein/excipient ratio, the experimental results showing good agreement with the predicted values. The resulting agglomerates were nearly spherical and the yield in the range between 250 and 1000 μm was over 80% (w/w). Also, it was possible to agglomerate recombinant bovine α_1^1-interferon as a model for a bioactive protein to spherical agglomerates with a good yield. The agglomeration behaviour of interferon was similar to that of BSA. It could be shown that the procedure of agglomeration using *n*-hexane has no influence on the biological activity of this protein.

Introduction

In the pharmaceutical industry, formulations containing bioactive proteins often present problems. These kinds of drugs are normally administered by injection but are often not stable in aqueous media. Therefore, almost all the formulations with bioactive proteins are prepared as lyophilisates. Normally, lyophilised powders are not produced in bulk, because milling would destroy the three-dimensional structure which is important for a fast disintegration. In addition, lyophilised powders are cohesive and not free flowing and therefore difficult to bottle with good accuracy. Lyophilisation is very expensive and consumes a lot of energy and space.

The goal of this work was to develop a low-cost alternative to the lyophilisation for the formulation of bioactive proteins to allow storage in dry state and provide a fast disintegration time. Therefore, spherical agglomeration technique was used. With the wet spherical agglomeration technique fine particles suspended in a liquid referred to as suspension liquid (SL) can be ag-

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glomerated by a small amount of a second immiscible liquid called bridging liquid (BL). The BL preferentially wets the particles and causes them to form agglomerates (Kawashima and Capes, 1974). This technology has been mainly developed in Canada for the selective agglomeration of coal (Sirianni et al., 1969) and was introduced in pharmaceutical research by Kawashima and Capes (1974). Generally, there are three possibilities to agglomerate a drug in this way. The first one is to suspend a hydrophilic drug in an organic solvent and agglomerate it by adding an aqueous BL (Kawashima et al., 1981). Also, it is possible to dissolve the drug in a three-component mixture (i.e., water/ethanol/chloroform). By adding one of these components to the mixture the drug can be crystallised and agglomerated by one of the other components (Kawashima et al., 1982). The third possibility is to suspend a lipophilic powder in a hydrophilic SL and add an organic BL (Amann, 1990).

The spherical agglomeration technique has not been described in the literature for the formulation of bioactive proteins so far. Bioactive proteins for pharmaceutical use are normally hydrophilic and soluble in water. It is, therefore, advantageous to use a very lipophilic organic solvent as suspension liquid. The solubility of the protein in this solvent should be very low. That ensures that the probability of wetting the protein particle is not very high. A low miscibility of the solvent with water is essential to ensure a low level of solvent residual in the protein. The BL can be an aqueous buffer solution with a pH that guarantees good stability of the protein used.

In a first step the critical parameters of the process were evaluated with a low-cost protein. Subsequently the influence of the process on the biological activity was tested with a second, bioactive protein.

Materials and Methods

Materials

Bovine serum albumin (BSA), fraction V (Sigma no. A-4503) was used as a model for a water soluble protein with a intermediate molecular weight and recombinant bovine α_1^1-interferon was used as a model for a bioactive protein. As excipient for a fast disintegration time mannitol (Hefti AG no. 5057) was used. All these powders were passed through a 125 μm sieve prior to use. The resulting median particle diameters were 60 μm for BSA, 45 μm for interferon and 60 μm for mannitol. In addition, BSA and mannitol were milled with an airmill (Haese, 1991). The resulting median particle diameters were 9 μm for BSA and mannitol. As suspension liquids *n*-hexane (Merck no. 8222280) and ethyl acetate (Merck no. 9623) were used. The suspension liquid can be recycled by distillation. As BL 0.1 M Tris/HCl buffer with a pH of 7.4 was used for the agglomeration of BSA and 0.1 M succinate buffer with a pH of 4.5 was used for the agglomeration of α_1^1-interferon.

Methods

Agglomeration process

The powders sieved (4.0 g) were added to a glass vessel with a six blade agitator. The entire system was kept at constant temperature (25°C) by a water bath. After 1 min agitation 100 ml suspension liquid were added. After 5 min of agitation the BL was sprayed into the suspension by a titration pump (Methrom 645 Multidosimat) using a laboratory-made nozzle with an HPLC capillary with a inner diameter of 0.1 mm. The agglomeration time was 10 min. The resulting agglomerates were filtered and dried in vacuum over silica gel and a special charcoal (FA. Dräger Aktivkohle Type Alcarbon). The latter was for removing residuals of suspension liquid from the agglomerates. After 72 h drying the agglomerates were classified by screening.

Particle size distribution

The particle size distribution was determined by sieving. The following parameters were described: median diameter, mean diameter, the fraction of fine (< 250 μm) and coarse (> 1000 μm) particles and the width of the distribution as the quotient of the particle sizes corresponding to a cumulative fraction of 75 and 25%. The yield of the agglomeration was defined as the fraction

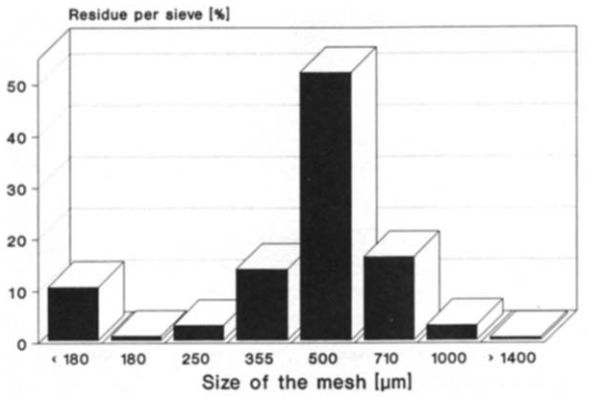

Fig. 1. Example of the size distribution of agglomeration at the optimal amount of BL using *n*-hexane as BL.

between 250 and 1000 μm. A typical particle size distribution is shown in Fig. 1.

Disintegration time

Five samples each with 30 mg agglomerates of the fractions 250–500 and 500–1000 μm were scattered on the liquid surface in five cylindrical beakers filled with 10.00 ml distilled water. The water was stirred with a magnetic stirrer at a stir rate which was adjusted to guarantee laminar conditions in the glass. The disintegration time was determined visually.

Geometrical properties of the agglomerates

The geometrical properties of the agglomerates were determined with an image processing system (Kontron Videoplan 2). 300 particles of the 250–500 and 500–1000 μm fractions were run over with an optical pen. The system determines the smallest (D_{min}) and the largest (D_{max}) diameter of each individual particle. A parameter R was developed (Eqn 1), which describes the roundness of the particles independently of the size of the particle. A value of R near 1.0 is indicative of a perfectly spherical agglomerate. An investigation with highly round paper stickers of the same size as the particles on the photograph showed that the highest mean value of R attainable with this method was 0.90. Fig. 2 shows

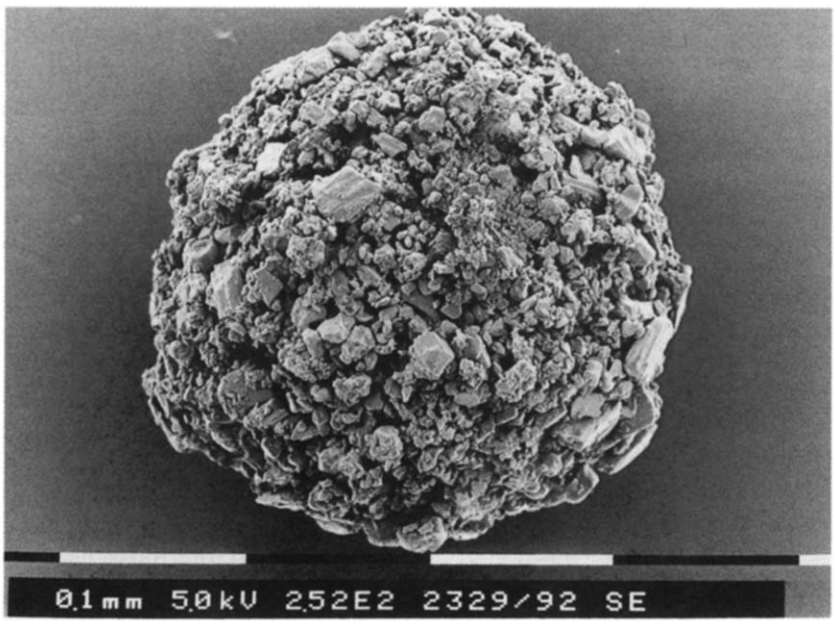

Fig. 2. Electron microscopic photograph of an agglomerate containing BSA and mannitol in the ratio of 1:1 using *n*-hexane as SL. The bar corresponds to 100 μm.

an agglomerate containing BSA and mannitol in the ratio of 1:1 using *n*-hexane as SL.

$$R = 1 - 2 \cdot \frac{(D_{max} - D_{min})}{(D_{max} + D_{min})} \quad (1)$$

Solvent residual in the agglomerates

The residuals of the SLs *n*-hexane and ethyl acetate in the agglomerates containing BSA were determined by a 'head space' gas chromatography method.

Content of BSA in the agglomerates

The content of BSA in the agglomerates was determined by a modified bromcresol green method (Uldall, 1984). This method is used in clinical chemistry as a specific assay for albumin in blood. Exactly 2.00 ml bromcresol green reagent containing 120 mmol bromcresol green in succinate buffer pH 4.2 were mixed with 2.00 ml aqueous BSA solution. The absorbance of the mixture was measured spectrophotometrically (Beckmann DU 64) against bromcresol green reagent at 630 nm after 1 min. The determined content was compared with the theoretical content in the agglomerates.

Determination of biological activity

The biological activity of the recombinant bovine α_1^1-interferon was determined by a cytopathic effect-inhibition assay.

Results and Discussion

Firstly, the influence of various process parameters was evaluated using BSA as a model protein and *n*-hexane as SL. The parameters discussed below were shown to affect the quality and the physical behaviour of the resulting protein agglomerates.

Amount of bridging liquid (BL)

The amount of BL is the most important parameter of the agglomeration process (Sastry and Fürstenau, 1977). In a first step, an optimal amount of BL could be defined. A quantity of BL smaller than the optimum results in a large fraction of fine particles. A quantity of BL larger than the optimum results in a great fraction of very coarse particles and an increasing tendency to form secondary agglomerates. In this case the fraction of fine particles is very small and the median diameter increases considerably. At the optimum of BL the yield is maximised (Fig. 3).

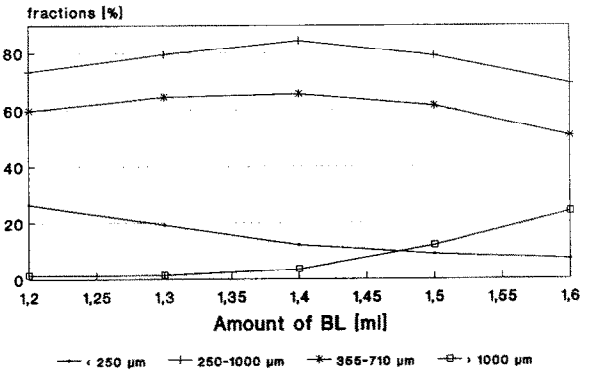

Fig. 3. Example of the determination of the optimal amount of BL using the system BSA/mannitol 1:1 and *n*-hexane as SL.

At the optimal amount of BL the resulting median diameter is a characteristic parameter for the agglomeration. This diameter depends only on the nozzle used (0.1 mm diameter, 580 μm and 0.18 mm diameter, 950 μm using the optimal amount of BL) and the size of the starting powder (sieved 580 μm and milled 500 μm).

Influence of excipient on the properties of the agglomerates

The disintegration time of the agglomerated BSA could be reduced by mixing the protein with mannitol before agglomeration. The resulting optimal amount of BL for the agglomeration of a mixture BSA/mannitol 1:1 indicated that mannitol consumes next to no BL for wetting (optimal amount of BL: 100% BSA, 2.7 ml; 50% mannitol, 1.4 ml).

It is, therefore, possible to assess the optimal amount of BL of each mannitol/BSA ratio by linear interpolation based on the optimal amount of BL in the case of 100% BSA. The resulting median diameters corresponding to the theoreti-

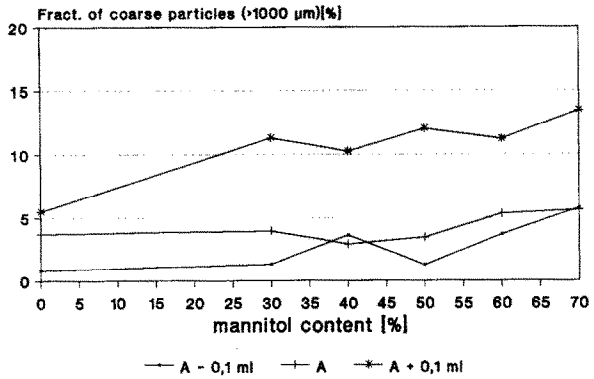

Fig. 4. Fraction of coarse particles of the agglomerates consisting of different mannitol contents using the amounts A, $A+0.1$ ml and $A-0.1$ ml as BL.

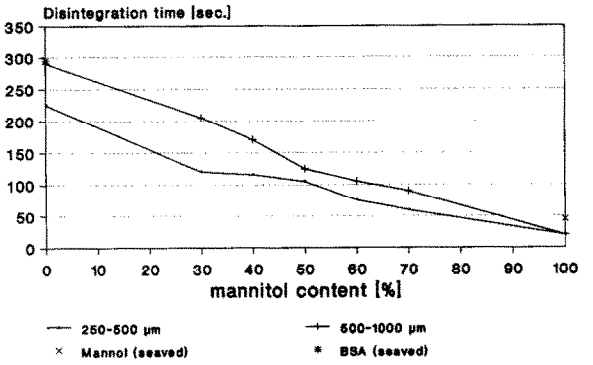

Fig. 6. Influence of mannitol content on the disintegration time.

cally predicted optimal BL for different mannitol/BSA ratios correlates very well with the experimental results. The experimental optimum was determined by agglomeration at the theoretically optimal amount A and $A+0.1$ ml and $A-0.1$ ml by checking the fractions of fine (<250 µm) and coarse particles (>1000 µm) (Fig. 4) and the yield of agglomeration (250–1000 µm). A nearly constant median diameter over the entire range of mannitol content indicates a good correlation with the theory (Fig. 5). Using 100% mannitol it was possible to form agglomerates with a comparable median diameter with only 0.1 ml BL.

Fig. 6 shows the disintegration time of agglomerates consisting of various ratios of mannitol/BSA for the fractions 250–500 and 500–1000 µm. It could be shown that the disintegration time strongly depends on the amount of added mannitol and the size of the agglomerates. The formed agglomerates containing pure protein or pure excipient dissolve faster than the starting substances.

The content of protein in the agglomerates depends on the amount of BL. Previous workers showed (Kawashima et al., 1981) that it is possible to agglomerate only one component of a powder mixture. If there is insufficient BL, the mannitol in the starting mixture is preferentially agglomerated. This is the reason why the content of protein in the fraction 250–1000 µm was found to be too small in this instance. In such a case the protein accumulates in the fraction of fine particles. At the optimal amount of BL, however, the content of protein is over 99% (w/w) in the desired range of 250–1000 µm. Using a nozzle with a greater inner diameter, the size of the droplet increases and the content of protein in the agglomerates decreases. The reason for this is the rapid agglomeration of mannitol near the large droplets. The protein therefore remains in the fraction of fine particles (Fig. 7).

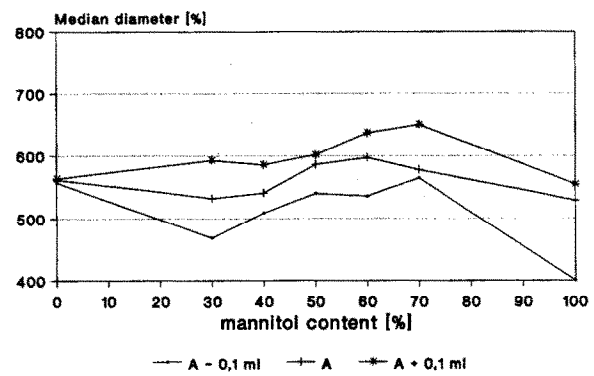

Fig. 5. Median diameter of the agglomerates consisting of different mannitol contents using the amounts A, $A+0.1$ ml and $A-0.1$ ml as BL.

Factorial design (2^4)

The influence of further parameters on the agglomeration were tested using a statistical factorial design (Retzlaff et al., 1975). For this purpose a 1:1 mixture of BSA/mannitol was agglomerated using the optimal amount of BL of

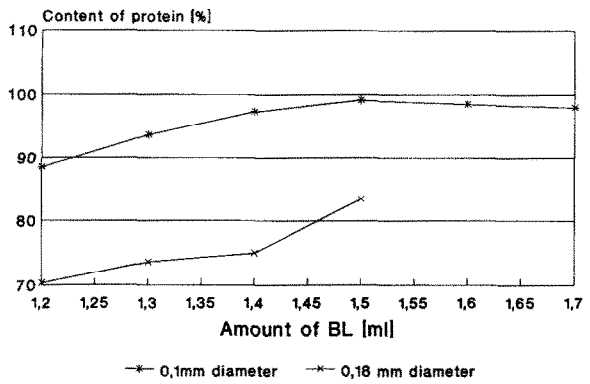

Fig. 7. Influence of the nozzle used on the content of protein in the agglomerates (500–1000 μm).

1.4 ml. Table 1 lists the parameters and the niveaus chosen used in this investigation.

As experimental error the three- and four-fold interactions were used (Sucker et al., 1991). The effects and interactions were tested at the significance levels 95 and 99%.

The resulting 16 agglomerations were carried out in a computer-randomised order (Table 2). The prepared agglomerates were tested as described in Materials and Methods and the calculated effects and interactions are given in Table 3.

The greatest influence on the result of agglomeration is exerted by the setting of the balance between the binding forces of the agglomerates and the shear stress of agitation. This can be affected by a long agglomeration time and high temperature. In this case the fraction of fine and coarse particles can be minimised and, therefore, the yield maximised. Also, more spherical agglomerates are obtained.

TABLE 1

Niveaus of the parameters tested in the factorial design

(A) Agitation speed (U/min)	−675
	+900
(B) Agglomeration time (min)	−5
	+10
(C) Spraying rate (ml/min)	−2.5
	+5
(D) Temperature (°C)	−25
	+35

Factor: (−) lower niveau; (+) upper niveau.

TABLE 2

Yates scheme with the tested combinations of niveaus (Sucker et al., 1991)

	Order of experiments	Factor			
		A	B	C	D
(1)	12	−	−	−	−
a	9	+	−	−	−
b	15	−	+	−	−
ab	5	+	+	−	−
c	11	−	−	+	−
ac	16	+	−	+	−
bc	1	−	+	+	−
abc	6	+	+	+	−
d	8	−	−	−	+
ad	10	+	−	−	+
bd	2	−	+	−	+
abd	4	+	+	−	+
cd	14	−	−	+	+
acd	3	+	−	+	+
bcd	13	−	+	+	+
abcd	7	+	+	+	+

Fine agglomerates with a small fraction of coarse particles can be produced at high agitation speed and high spraying rate. A high content of protein in the agglomerates can be achieved by a high spraying rate using the optimal amount of BL. Small droplets of BL can agglomerate the powder mixture more homogeneously.

All the tested parameters had no influence on the disintegration time. It appears that this time is only affected by the powder composition and the size of the agglomerates (Fig. 6).

Suspension liquid

An ideal SL for the agglomeration of water soluble proteins is *n*-hexane. The resulting agglomerates were nearly spherical ($R = 0.85$–0.9) and free flowing. The yield of agglomeration was very good (over 80% in the fraction 250–1000 μm). Ethyl acetate, on the other hand, has a considerable solubility in water and is more hydrophilic than *n*-hexane. The resulting agglomerates are not spherical (R 0.6–0.65) and are very voluminous. The determined optimum of the amount of BL was very high (5.3 ml for agglomeration of 4.0 g BSA, as compared to 2.7 ml in *n*-hexane).

A possible explanation for this effect is the solubility of the BL in ethyl acetate. Agglomerations with water saturated ethyl acetate as SL showed that the increased consumption of BL compared to agglomeration in *n*-hexane is only due to the BL dissolved in the SL (Fig. 8).

Residual solvent in the agglomerates

The residual organic solvents measured in the agglomerates were in the order of 0.05%. Using charcoal in the drying procedure, the residual solvents could be reduced to less than 0.01%. No difference could be found between the residual ethyl acetate and *n*-hexane as SM.

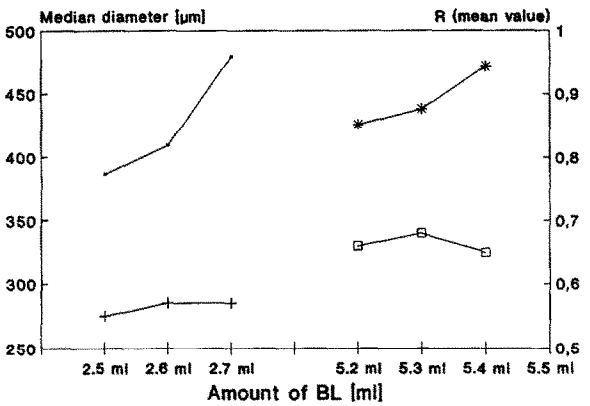

Fig. 8. Influence of the saturation of ethyl acetate as SL on the consumption of BL. (■) Median diameter, saturated; (*) median diameter, not saturated; (+) R (250–500 µm), saturated; (□) R (250–500 µm), not saturated.

TABLE 3

Significant influences and interactions on the result of agglomeration

Parameter/effect	Significance
(1) Yield (250–1000 µm)	
B^+ good yield at long agglomeration time	> 99%
D^+ good yield at high temperatureratur	> 99%
(2) Median value	
B^+ large particles at long agglomeration time	> 99%
C^- large particles at low spraying rate	> 99%
BD^+ interaction agglomeration time/temperature	95–99%
(3) Mean diameter	
C^- large particles at low spraying rate	> 99%
A^- large particles at high agitation speed	95–99%
(4) Fraction of fine particles (< 250 µm)	
B^- low fraction at long agglomeration time	> 99%
D^- low fraction at high temperature	> 99%
(5) Fraction of coarse particles (> 1000 µm)	
A^- low fraction at high agitation speed	95–99%
B^- low fraction at long agglomeration time	> 99%
C^- low fraction at high spraying rate	> 99%
D^- low fraction at high temperature	> 99%
BD^+ (B^-, D^-) interaction agglomeration time/temperature	> 99%
(6) Content of protein in the agglomerates (250–500 and 500–1000 µm)	
C^+ high content of protein at high spraying rate 500–1000 µm	95–99%
(7) Geometrical properities of the agglomerates (mean value of R (250–500 and 500 –1000 µm)	
B^+ spherical agglomerates at long agglomeration time	> 99%
D^+ spherical agglomerates at high temperature	> 99%

Agglomeration with bovine α_I^1-interferon

It was possible to agglomerate α_I^1-interferon to spherical agglomerates with a good yield (85% (w/w) in the fraction 250–1000 µm, $R = 0.88$). The behaviour of agglomeration is similar to that of BSA. It could be shown that the procedure of agglomeration using *n*-hexane had no influence on the biological activity of this protein.

Conclusions

With the system consisting of *n*-hexane as SL and an aqueous BL it was possible to prepare free flowing spherical agglomerates with a good yield. The disintegration time could be controlled by the excipient/protein ratio in a range which allows the use of the agglomerated product as a rapidly soluble parenteral dosage form. In contrast to lyophilisation, it is possible to agglomerate proteins in a batch and bottle them with good accuracy. The agglomerates could be produced within a short time and with low energy cost. As shown with α_I^1-interferon the process has no influence on the biological activity of this protein.

Acknowledgements

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