The Use of Bioneedles in Vaccine Storage: a Hot Topic or Cold Case?

D.F.A. Kaal

12-07-2024

Abstract

Most vaccines must be stored at low temperatures to keep their effectiveness. Because this requires cold transport and storage, which is expensive and not always reliable, it is of interest that an alternative solution be found. The Bioneedle is a small needle made from a biodegradable polymer that can be filled with a vaccine and transported and stored outside the cold-chain. This bachelor thesis aims to investigate the stabilizing effect of the Bioneedle material on Bovine Serum Albumin for a short period at extreme temperatures. The retention of the structure in solution and Bioneedle samples was investigated using CD spectroscopy and FT-IR spectroscopy. Although the preparation of Bioneedles caused degradation, the samples stored in Bioneedles showed less degradation when exposed to high temperatures. While the analytical methods used do provide some insight in assessing the quality of BSA, more research is required to conclusively say if it can be used as a model protein.

De meeste vaccinaties moeten op een lage temperatuur bewaard worden om hun effectiviteit te behouden. Omdat gekoeld vervoer en opslag hierdoor vereisten zijn, en dit niet goedkoop of overal beschikbaar is, is het vinden van een alternatief van belang. Een Bioneedle is een kleine naald gemaakt van biologisch afbreekbaar materiaal, die gevuld kan worden met een vaccin en niet gekoeld bewaard hoeft te worden. Deze bachelor scriptie heeft als doel om het stabiliserende effect van het Bioneedle materiaal op runderalbumine te onderzoeken voor een korte periode op hoge temperaturen. Hoe goed de structuur intact bleef werd onderzocht door middel van CD spectroscopie en FT-IR spectroscopie. Hoewel het toevoegen van het runderalbumine aan de Bioneedle voor wat afbraak zorgde, bleef het runderalbumine stabieler in de Bioneelde tijdens het testen op verhoogde temperaturen. Hoewel de analytische methoden die hier gebruikt zijn wel een inzicht geven in het behoud van de structuur van runderalbumine, is er meer onderzoek nodig om te kunnen bepalen of het kan worden gebruikt als model proteïne.

1 Introduction

Since the invention of vaccines they have played an important role in maintaining and improving public health by preventing or even wiping out infectious diseases. With widespread adaption of vaccination programs for diseases such as measles, smallpox and polio the morbidity and mortality of these illnesses have been greatly reduced, preventing millions of deaths every year [1]. However there are still many areas where access to vaccines is inadequate causing many people not to receive immunization against preventable illnesses. The WHO has set goals in the Immunization Agenda 2030 to achieve a 90% coverage for essential vaccines and to provide all countries with an adequate supply of affordable vaccines of assured quality [2]. One challenge that must be faced is the storage and transportation of vaccines. This generally requires a cold chain which is often less prevalent in developing or war-torn countries, especially in remote locations [2]. Creating vaccines that do not require a cold chain should prove useful in the efforts of WHO.

Vaccines can be categorized into different types depending on composition and type of immunological mechanism utilized to induce protection. Some vaccines use weakened or inert forms of the pathogen while others, like subunit and recombinant vaccines, contain specific proteins of the pathogen to stimulate the body's immune system [3]. The Hepatitis B vaccine, HPV vaccine and some influenza vaccines all make use of such proteins. When these vaccines are exposed to high temperatures the proteins start to denature, this leads to changes in their structure and biological activity [4]. This denaturation disrupts the three dimensional structure of the protein, which is crucial for causing an immune response that leads to immunization. Current methods used to improve temperature stability of protein vaccines are lyophilization (freeze-drying), and the addition of stablizers.

As mentioned above, one method of stabilizing proteins is lyophilization. Generally solid-state is the preferred choice of storage method for protein therapeutics to improve stability and preserve biological activity [5]. To create solid-state proteins lyophilization (freeze-drying) is used. This is done by first freezing a protein solution, then reducing the pressure to allow the frozen water to go directly from solid to gas phase and evaporate. However these processing steps can expose proteins to various

stress conditions, leading to denaturation.
During cooling, the protein solution starts to freeze. In this process ice nucleation occurs and crystal growth starts, creating an ice-water interface. This interface can affect the stability of proteins due to interaction and adsorption of protein molecules at the ice-water interface [5]. One way to minimize the effects on the ice-water interface is by using a low cooling rate. This is because a low cooling rate produces large ice crystals with a lower surface area compared to the many small ice crystals that form with a high rate of cooling [6].
Another problem that occurs during the freezing process is pH shift. Proteins are stable in pH range (6-7), if the pH changes outside of this range the protein undergoes denaturation or unfolding. Therefore protein solutions are often stored in buffers to ensure a stable pH. When inorganic salts are used as a buffer, one ionized species can have a different freezing point than the non-ionized species. This difference leads to the freezing of one form before the other during lyophilization. This has been linked to drastic changes in pH in the liquid medium, causing denaturation [7].

Another method used to stabilize protein vaccines is by using stabilizing agents. One type of stabilizer is sugars and polyols like trehalose and glycerol. These compounds have been shown to stabilize proteins and reduce the effects of increased temperature on denaturation [8, 9]. These compounds also possess cryoprotective properties, which allow them to be used in lyophilization to reduce the effect of the stresses caused in this process [5]. However, the addition of such compounds makes the production of vaccines more complicated and therefore more expensive.

One way to mitigate the issues is to change the way vaccines are administered. Instead of injecting liquid vaccines using a needle and syringe, Bioneedles can be used [10]. Bioneedles are small hollow mini-implants fabricated from biodegradable polymers that can be filled with 4,0-4,5μl of liquid medicine formulations. The Bioneedle absorbs the medicine which will bind to the structure of the needle. Because of this embedding the movement of molecules is inhibited leading to a decrease in physical en chemical denaturation. This means the stability of vaccines is increased, even without freeze-drying. This does however not mean that freeze-drying is redundant. The removal of water further increases the stability of the vaccine and the protective environment of the Bioneedle could alleviate the stresses that cause denaturation during the freeze-drying process.
The Bioneedles can be subcutaneously injected using compressed air. Once beneath the skin the Bioneedle dissolves and the medicine is reconstituted by the body fluids and is released. Because this process does not use conventional needles and syringes there is no need for disposal of such items and there is no risk of cross-contamination and needle-stick injuries.

Bioneedles have already been shown to increase storage stability for several vaccines [11, 12, 13]. However, Bioneedle Drug Delivery is interested in studying the stabilization mechanism in detail with use of a protein as a model system. If the model protein can retain its structure the effect of processing and storage variables can be studied at length, without the need for costly vaccine proteins. The protein that will be used is the widely available Bovine Serum Albumin (BSA). The properties of this protein have been studied well. BSA is a globular protein with a molecular weight of approximately 66KDa consisting of a single polypeptide chain of 583 amino acids. The proteins secondary structures are estimated to be 54% α-helix and 18% β-forms [14]. BSA remains stable in a wide range of pH (4-9) [14] and and has a relatively high temperature stability, it is also a moderately sized protein with good solubility which aids in the use of various analytical techniques. These qualities combined mean that BSA is an ideal candidate for experimenting on the factors that influence temperature stability.

The use of Bioneedles and their promising qualities has lead to the following research question: **"How does embedding Bovine Serum Albumin (BSA) in Bioneedles influence its temperature stability?"**
To answer this question the following subquestions need to be answered:

1. What analysis techniques can be used to asses the quality of the BSA?

2. What is the effect of the Bioneedle on the temperature stability of BSA?

3. What is the effect of loading BSA into the Bioneedle on the structure of reconstituted BSA?

Two methods that can be used to evaluated the structure of BSA are Circular Dichroism (CD) spectroscopy [15] and Fourier-Transform Infrared (FT-IR) spectroscopy [16]. The signal CD spectroscopy produces is highly dependant on the secondary structure of the protein. The α-helix and β-sheets both produce signature characteristics in the spectrum. This is a highly sensitive technique and does not require a high protein concentration or large amounts of sample to provide accurate measurements. The secondary structure of proteins can also be measured via FT-IR spectroscopy. Each type of secondary structure influences the absorption frequencies of the C=O bond and change the shape of the Amid I band (1700cm^{-}1 - 1600cm^{-}1). An Attenuated Total Reflection (ATR) module was chosen because of its high sensitivity and ease of sample preparation. Measurements on proteins using FT-IR are usually done using relatively high protein concentrations (< 10mg/ml), however some papers show that a readable spectrum can be produced with protein concentrations as low as 1mg/ml.

2 Methods

The BSA was purchased from Sigma-Aldrich. The Bioneedles were provided by Bioneedles Drug Delivery BV (Leiden, The Netherlands). All solutions for the experiments were prepared with Milli-Q water.

2.1 Bioneedle production

For the production of the Bioneedles a thermoplastic biopolymer is used. This is made by blending the individual components in an extruder and bringing them to the right temperature and hydration. This is then cooled and cut into small pellets. This material can then be shaped into the shape of the needle using injection molding. This results into the final product of a Bioneedle, show in figure 1.

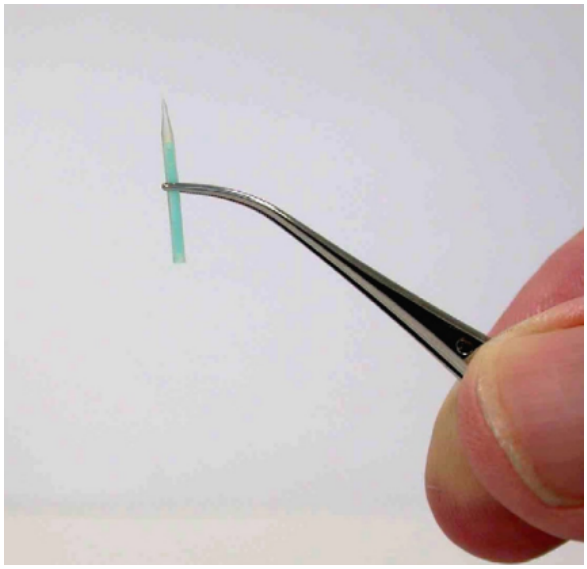

Figure (1) *A Bioneedle held between tweezers.*

2.2 Sample preparation

Samples of both BSA in solution and in Bioneedle were subjected to a range of temperatures from 60°C to 90°C with increments of 10°C for one hour. For each temperature increment a fluid sample and a Bioneedle sample were prepared. Because CD spectroscopy and FT-IR spectroscopy require different protein concentrations, different samples were prepared for each analytical method. All BSA quantities came from the same 10mg/ml solution.

2.2.1 CD spectroscopy samples

For every condition involving Bioneedles 3 needles were used. Each of these Bioneedles were loaded with 5μl of the 10mg/ml solution and allowed to dry for a few hours at ambient temperature before use. After exposure to high temperature for one hour the three Bioneedles were placed in 1,5ml of water to reconstitute the BSA for a final concentration of 0,1mg/ml. For measurements involving liquid samples the 10 mg/ml solution was diluted to 0,1mg/ml in one large batch and 1ml of this solution was placed in a vial for every individual measurement.

2.2.2 FT-IR spectroscopy samples

Because a limited amount of Bioneedles were available and creating samples for FT-IR analysis is resource expensive only the low and high end of the temperature range were recorded using this method. For every condition involving Bioneedles 10 needles were used. Each of these Bioneedles were loaded with 10μl of the 10mg/ml solution and allowed to dry for a few hours at ambient temperature before use. After exposure to high temperature for one hour the five bioneedles were placed in 1ml of water to reconstitute the BSA for a final concentration of 1mg/ml. For every condition involving fluid samples the 10mg/ml BSA solution was diluted to 1mg/ml in one batch, and 1ml of placed in a vial for every measurement.

2.3 CD spectroscopy

A Jasco J-1500 CD spectrometer was used for the circular dichromism measurements, using a 1 mm path length. For each measurement the average of 3 runs was used. A measurement of water was made and used as a baseline, which was automatically subtracted. The range for each measurement was 185-260nm with a resolution of 0,2nm. For further analysis the spectra were plotted using Origin and smoothed using the Savitzky-Golay method with a 10 point window to reduce noise. To determine the secondary protein structures these smoothed spectra were loaded onto DichroWeb [17, 18] and analysed using CDSSTR[19] with reference set 6[20]. This method calculates the secondary structure of a protein by assuming the CD spectrum can be approximated using a linear combination of the reference proteins. These reference proteins each contain different proportions of α-helix β-forms, and random coils. Reference set 6 specifically was chosen because it contained globular proteins and partially degraded proteins [18], which align with the measurements taken here. The HT data was also recorded to account for changes in protein concentration. This was done by taking the peak voltage from each measurement (all at 185nm), and removing the HT amount recorded for water at this wavelength. The spectrum for the master solution of BSA was set as 1. If a value differed from the HT value of the master solution the the according spectrum was multiplied by the same factor as the difference in HT data.

2.4 FTIR spectroscopy

For fourier transform IR analysis a Bruker Alpha FTIR spectrometer equiped with an ATR module was used. For each measurement the spectrum the data between 4000 cm^{-1} and 400 cm^{-1} was recorded with a resolution of 2 cm^{-1}. For each measurement 512 scans were

recorded and averaged. The background spectrum was first recorded and subtracted automatically. The spectrum of water was also registered and subtracted. The absorption spectra were smoothed using FFT filtering and the amid I band (1700cm$^{-}1$ - 1600cm$^{-}1$) was analysed. To estimate the secondary protein structure it is assumed that proteins can be considered as a linear sum of definite fundamental secondary structural elements (helix, sheet, turns and unordered) [21]. The minimum of the second derivative determined the centre of Gaussians, while the area of the Gaussians determined the contribution from each structural element. The secondary structure assigned to each Gaussian was done according to the method reported by Dong et al [22].

3 Results

3.1 Reconstitution of BSA from Bioneedle

When prepairing samples the BSA is added to the Bioneedle and allowed to dry. To measure how well the BSA is preserved during this process we compare the CD spectra of BSA from the master solution to the CD spectra of BSA that was reconstituted from a needle after it dried. In figure 2 it can be seen that the BSA spectra from the Bioneedle maintains the same shape as the spectra from master solution, however the intensity is decreased. To account for differences in concentration the CD spectra were multiplied by a factor according to the peak HT values of the measurement.

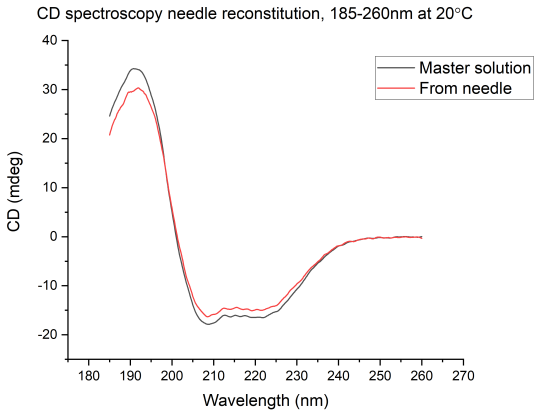

Figure (2) *CD spectroscopy of the BSA master solution and of BSA reconstituted from a Bioneedle. The spectra have been smoothed using the Savitzky-Golay method and equalized for concentration based on HT.*

3.2 Stability of BSA in high temperatures

The BSA in solution and in Bioneedles were tested at a few different temperatures from 50°C to 90°C for one hour. Due to high evaporation at 90°C the BSA in solution dried up and could not be used. In figure 3 the CD spectra of the BSA in solution is shown. After only one hour a clear difference is visible showing that the BSA in solution quickly starts to lose the shape of the master solution CD spectra.

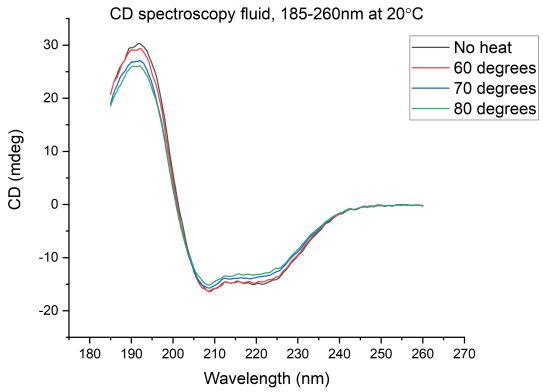

Figure (3) *CD spectroscopy of BSA in solution after temperature exposure. The spectra have been smoothed using the Savitzky-Golay method and equalized for concentration based on HT.*

The BSA in Bioneedles was exposed to the same conditions. As seen in figure 4 the CD spectra of the BSA in the Bioneedles also somewhat starts to lose its shape, however to a much lesser extent than the CD spectra of the BSA in solution.

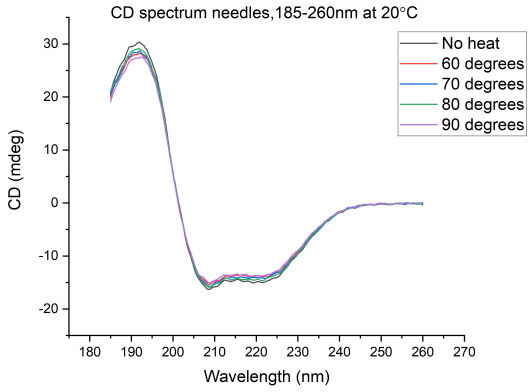

Figure (4) *CD spectroscopy of BSA in Bioneedle after temperature exposure. The spectra have been smoothed using the Savitzky-Golay method and equalized for concentration based on HT.*

Each of the CD spectra has a peak maxima at or around 190nm these peak maxima were plotted as a function of the temperature they were exposed to. As seen in figure 5 the peak maxima in the CD spectra of the fluid BSA samples show a clear decrease when the temperature they were exposed to is increased. With the peak maxima of the BSA in Bioneedles this relation is less clear and the peak maxima even increases slightly before also going down after exposure to the highest temperature.

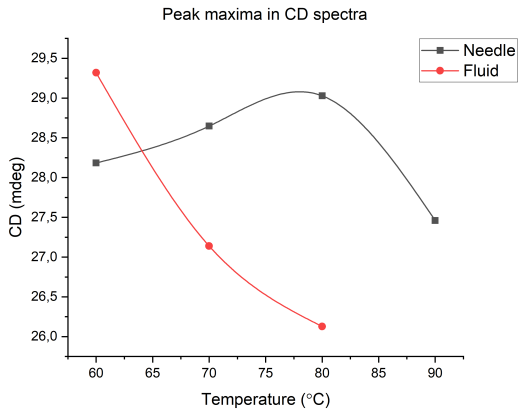

Figure (5) *Peak maxima of the CD spectra as a function of exposed temperature.*

The secondary protein structure was calculated on DichroWeb using the CDSSTR method with reference set 6 [18]. This resulted in the secondary structure composition as shown in table 1. All calculations showed a NRMSD$\leq 0,02$. The full results from DichroWeb can be found in the appendix3.

Table 1 shows that the BSA in solution denatures more with more temperature exposure. The helix structures start to break down resulting in a higher count of turns and unordered secondary structures proving that the BSA denatures. After exposure to 80 °C the α-helix content of the fluid BSA samples goes from 57% to 51%, a decrease of 10,5%, while the unordered content goes from 23% to 28%, an increase of 21,7%. The α-helix content of the Bioneedle samples goes from 56% to 55%, a decrease of 1,8%, while the unordered content goes from 23% to 24%, an increase of 4,3%. This shows that while some degradation occurs during Bioneedle preparation, after this process the BSA in Bioneedles is over 5 times more resistant to degradation compared to BSA in solution.

Table (1) *Secondary protein structure of BSA calculated from the CD spectra using CDSSTR with reference set 6 on DichroWeb.*

Sample	α-Helix	β-Sheets	β-Turns	Unordered
BSA master	57%	8%	11%	23%
Fluid 60 °C	57%	7%	12%	25%
Fluid 70 °C	52%	8%	12%	28%
Fluid 80 °C	51%	9%	13%	28%
Needle no heat	56%	8%	13%	23%
Needle 60 °C	55%	8%	13%	24%
Needle 70 °C	55%	7%	13%	24%
Needle 80 °C	55%	7%	12%	24%
Needle 90 °C	54%	8%	13%	25%

Some conditions were also measured using FTIR spectroscopy. When the liquid sample for 90°C was fully evaporated a new sample was prepared and the experiment was done at 80 °C. For each measurement the the background and water spectra were subtracted. The resulting absorption spectra did show peaks in the Amide I and II bands, however the difference between the sample spectra and water spectra was very low ($<$0,005 a.u.) and the signal was very noisy. The absorption spectra were smoothed by FFT filtering and the amid I band (1700cm$^{-}1$ - 1600cm$^{-}1$) was analysed. Estimation of secondary protein structure was done by assuming the second derivative of the spectra were the centre of a Gaussian that can be assigned to secondary structural element and that the area of the Gaussian determined its contribution [21]. The secondary structure assigned to each Gaussian was done according to the method reported by Dong et al [22]. If a Gaussian did not align within these ranges it was assigned as undefined. This resulted in the secondary structure compositions shown in table 2.

Table (2) *Secondary protein structure of BSA calculated from FTIR spectra.*

Sample	α-Helix	β-Sheets	β-Turns	Unordered	Undefined
BSA master	54%	30%	2%	0%	12%
Fluid 60 degrees	55%	21%	5%	0%	19%
Fluid 80 degrees	49%	28%	0%	0%	23%
Needle 60 degrees	59%	22%	3%	0%	16%
Needle 90 degrees	50%	31%	8%	0%	11%

The secondary protein structure calculated from the FTIR spectra varies greatly and large parts of the structure are unknown. This is likely due to the low signal strength and large amount of noise.

4 Discussion and conclusion

The results show that Bioneedles help stabilize BSA, especially during high temperature exposure. The BSA that was stored in Bioneedles retained more of its structure compared to the BSA that was kept in liquid form after one hour of extreme temperature exposure. The process of loading the BSA onto the Bioneedle also seems to cause some degradation, likely due to the drying. This means that when using Bioneedles for vaccine application some losses occur during the loading process, but afterwards the protein degrades less with temperature exposure. These factors should be considered before using Bioneedles to stabilize vaccines. However in general, where vaccines are made in bulk and transported to stockpiles, and to move away from dependence on the cold-chain, Bioneedles show promising temperature stabilizing effects.

Because these test were only performed with an hour of temperature exposure the degradation of the protein are mostly only visible at the highest temperature range. While this does show a clear effect in the stabilizing properties of the Bioneedle, to be able to better quantify and investigate the effects of the Bioneedle a longer timeframe should be used. When this is done the degradation difference of BSA in solution compared to in a Bioneedle at lower temperatures can be investigated. This would also provide more insight into the real world application of

Bioneedles, where the temperature outside of the cold-chain would generally be around 20-30°C, and storage time could be months or years.

The results gathered by CD spectroscopy show a clear difference in the stability when the BSA was stored in the bioneedle compared to BSA in solution both visually from the produced spectrum and from the secondary structure calculated. However, CD spectroscopy only provides insight in the total amount of secondary structures present in the sample, while this does provide some insight, it does not directly correlate with the amount of intact protein molecules in the sample. If for example all BSA molecules are only slightly degraded it would show the same spectrum as if a small amount of BSA molecules degraded completely. While these situations would show similarly on the CD spectra, the effectiveness of the vaccine differs drastically.

While the results gathered using FT-IR spectroscopy suffer from the same previously mentioned shortfall, the results gathered in this experiment do not prove useful in determining the stability of BSA for another reason, the high amount of noise. While some experiments have measured protein structure at protein concentrations of 1mg/ml, using the equipment and method laid out in this experiment the spectra contained too much noise to be able to asses the secondary structure in a proper manner. One way to mitigate this would be to increase the concentration of the samples.

In conclusion, the Bioneedle provides a stabilizing effect making BSA 5 times more resistant to high temperature exposure. While some degradation occurs during the drying process, the increased stabilization should outweigh this when to vaccine is stored for a long period of time. While analysis using CD spectroscopy does provide a useful insight in the retention of protein structure, it does lack the ability to measure the amount of fully intact protein, which is an important factor in determining the effectiveness of a vaccine after temperature exposure. FT-IR spectroscopy could potentially be used as a secondary factor to measure secondary protein structure when used with a higher concentration, or better equipment. However because this does not provide much more insight than CD spectroscopy, because they in essence measure the same thing using different methods, CD spectroscopy would be the better candidate. However, to fully investigate the usefulness of Bioneedles as vaccines, another method should be used to investigate the amount of fully intact protein after temperature exposure.

4.1 Recommendations

Further research is needed to corroborate the findings in this study. Before it can be concluded if Bioneedles provide the stabilizing effects needed for vaccine storage more insight into the amount of intact protein molecules is needed. One way this can be achieved is by using an enzyme as a model protein. By repeating the experiments done in this paper with an enzyme like horseradish peroxidase and performing an enzyme activity assay as well as CD spectroscopy more insight is achieved in how well CD spectroscopy predicts the amount of intact protein. This experiment would determine if CD spectroscopy alone could be used to analyse temperature stability.

In order to gain a better insight for the use of Bioneedles for long term vaccine storage these same test should also be performed in settings resembling real world conditions, where the vaccines stored outside the cold chain experience temperatures between 20°C and 30 °C for months or years.

Another factor that should be researched further is the preparation of the Bioneedle. One factor that should be considered is freeze-drying. When factors such as freezing speed, drying time and use of cryoprotectants are optimised the losses that occur during the drying process can be minimized. Therefore less starting product is needed to incur the same immunization effect in the vaccine. However even if freeze-drying does not improve the loss during the loading process, it should be considered regardless, because freeze-drying ensures the final vaccine is completely dry in order to prevent mold from growing during storage.

Before any findings using BSA as a model protein can be generalized, they also have to be tested on different proteins and eventually the real vaccines. Currently the possibility exists that the stabilizing properties of Bioneedles are due to a specific interaction between with BSA and do not extrapolate to different proteins. However because Bioneedles have improved shelf stability for other vaccines [11, 12, 13] it is likely that further research using a model protein can be generalized.

With this research the first step is taken to study the stabilization mechanism of Bioneedles with the use of a model protein. While more research is needed in order to determine if BSA is a good model protein, the insights found here are promising. Because the use of a model protein makes research into the stabilizing factors of Bioneedles faster and cheaper, the insights in this paper could prove to be of importance into reaching the goals set out by the WHO in the Immunization Agenda 2030.

References

[1] *WHO: Vaccines and immunization*. URL: https://www.who.int/health-topics/vaccines-and-immunization#tab=tab_1 (visited on 06/03/2024).

[2] *WHO: Immunization Agenda 2030*. URL: https://www.who.int/teams/immunization-vaccines-and-biologicals/strategies/ia2030 (visited on 06/03/2024).

[3] N Dumpa et al. "Stability of Vaccines". In: *AAPS PharmSciTech* 20.42 (2019). DOI: 10.1208/s12249-018-1254-2.

[4] P. Masson and S. Lushchekina. "Conformational Stability and Denaturation Processes of Proteins Investigated by Electrophoresis under Extreme Conditions." In: *Molecules* 27.20 (2022). DOI: 10.3390/molecules27206861.

[5] Arun Butreddy et al. "Instability of therapeutic proteins — An overview of stresses, stabilization mechanisms and analytical techniques involved in lyophilized proteins". In: *International Journal of Biological Macromolecules* 167 (2021), pp. 309–325. ISSN: 0141-8130. DOI: https://doi.org/10.1016/j.ijbiomac.2020.11.188. URL: https://www.sciencedirect.com/science/article/pii/S0141813020351242.

[6] Wei Wang. "Lyophilization and development of solid protein pharmaceuticals". In: *International Journal of Pharmaceutics* 203.1 (2000), pp. 1–60. ISSN: 0378-5173. DOI: https://doi.org/10.1016/S0378-5173(00)00423-3. URL: https://www.sciencedirect.com/science/article/pii/S0378517300004233.

[7] Sydney O. Ugwu and Shireesh P. Apte. "The effect of buffers on protein conformational stability". In: *Pharmaceutical technology* 28 (2004), pp. 86–108. URL: https://api.semanticscholar.org/CorpusID:43353395.

[8] Thomas HOTTIGER et al. "The role of trehalose synthesis for the acquisition of thermotolerance in yeast". In: *European Journal of Biochemistry* 219.1–2 (Jan. 1994), pp. 187–193. DOI: 10.1111/j.1432-1033.1994.tb19929.x.

[9] Dooha Kim and Yong J. Lee. "Effect of glycerol on protein aggregation: Quantitation of thermal aggregation of proteins from Cho cells and analysis of aggregated proteins". In: *Journal of Thermal Biology* 18.1 (Feb. 1993), pp. 41–48. DOI: 10.1016/0306-4565(93)90040-z.

[10] *Bioneedle Drug Delivery — Vaccination Technology Of The 21st Century*. URL: https://www.bioneedle.com (visited on 06/04/2024).

[11] Hoang J.H.B. Hirschberg et al. "Bioneedles as alternative delivery system for hepatitis B vaccine". In: *Journal of Controlled Release* 147.2 (2010), pp. 211–217. ISSN: 0168-3659. DOI: https://doi.org/10.1016/j.jconrel.2010.06.028. URL: https://www.sciencedirect.com/science/article/pii/S0168365910004761.

[12] Hoang J.H.B. Hirschberg et al. "Bioneedles™ as vaccine carriers". In: *Vaccine* 26.19 (2008), pp. 2389–2397. ISSN: 0264-410X. DOI: https://doi.org/10.1016/j.vaccine.2008.02.067. URL: https://www.sciencedirect.com/science/article/pii/S0264410X08002612.

[13] Peter C. Soema et al. "Solid Bioneedle-Delivered Influenza Vaccines Are Highly Thermostable and Induce Both Humoral and Cellular Immune Responses". In: *PLOS ONE* 9.3 (Mar. 2014), pp. 1–8. DOI: 10.1371/journal.pone.0092806. URL: https://doi.org/10.1371/journal.pone.0092806.

[14] *Sigma-Aldrich Product information sheet Bovine Serum Albumin*. URL: https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/277/289/a7030pis.pdf (visited on 06/07/2024).

[15] Adewale Olamoyesan, Dale Ang, and Alison Rodger. "Circular dichroism for secondary structure determination of proteins with unfolded domains using a self-organising map algorithm SOMSpec". In: *RSC Adv.* 11 (39 2021), pp. 23985–23991. DOI: 10.1039/D1RA02898G. URL: http://dx.doi.org/10.1039/D1RA02898G.

[16] Rui Lu et al. "Probing the secondary structure of bovine serum albumin during heat-induced denaturation using mid-infrared fiberoptic sensors". In: *Analyst* 140 (3 2015), pp. 765–770. DOI: 10.1039/C4AN01495B. URL: http://dx.doi.org/10.1039/C4AN01495B.

[17] *DichroWeb*. URL: http://dichroweb.cryst.bbk.ac.uk/html/home.shtml (visited on 06/07/2024).

[18] Andrew J. Miles, Sergio G. Ramalli, and B. A. Wallace. "DichroWeb, a website for calculating protein secondary structure from circular dichroism spectroscopic data". In: *Protein Science* 31.1 (2022), pp. 37–46. DOI: https://doi.org/10.1002/pro.4153. eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1002/pro.4153. URL: https://onlinelibrary.wiley.com/doi/abs/10.1002/pro.4153.

[19] Larry A. Compton and W.Curtis Johnson. "Analysis of protein circular dichroism spectra for secondary structure using a simple matrix multiplication". In: *Analytical Biochemistry* 155.1 (1986), pp. 155–167. ISSN: 0003-2697. DOI: https://doi.org/10.1016/0003-2697(86)90241-1. URL: https://www.sciencedirect.com/science/article/pii/0003269786902411.

[20] Narasimha Sreerama and Robert W. Woody. "Estimation of Protein Secondary Structure from Circular Dichroism Spectra: Comparison of CONTIN, SELCON, and CDSSTR Methods with an Expanded Reference Set". In: *Analytical Biochemistry* 287.2 (2000), pp. 252–260. ISSN: 0003-2697. DOI: https://doi.org/10.1006/abio.2000.4880. URL: https://www.sciencedirect.com/science/article/pii/S0003269700948802.

[21] K V Abrosimova, O V Shulenina, and S V Paston. "FTIR study of secondary structure of bovine serum albumin and ovalbumin". In: *Journal of Physics: Conference Series* 769.1 (Nov. 2016), p. 012016. DOI: 10.1088/1742-6596/769/1/012016. URL: https://dx.doi.org/10.1088/1742-6596/769/1/012016.

[22] J Kong and S Yu. "Fourier transform infrared spectroscopic analysis of Protein Secondary Structures". In: *Acta Biochimica et Biophysica Sinica* 39.8 (2007). DOI: 10.1111/j.1745-7270.2007.00320.x.

5 Appendix

Table (3) *Full secondary structure composition as produced by DichroWeb from the CD spectroscopy data.*

METING	Helix1	Helix2	Helix total	Strand1	Strand2	Strand total	Turns	Unordered	NRMSD
BSA master	0,37	0,2	0,57	0,05	0,03	0,08	0,11	0,23	0,015
Fluid 60 degrees	0,37	0,2	0,57	0,04	0,03	0,07	0,12	0,25	0,015
Fluid 70 degrees	0,34	0,18	0,52	0,04	0,04	0,08	0,12	0,28	0,016
Fluid 80 degrees	0,33	0,18	0,51	0,05	0,04	0,09	0,13	0,28	0,014
Needle 60 degrees	0,36	0,19	0,55	0,04	0,04	0,08	0,13	0,24	0,014
Needle 70 degrees	0,36	0,19	0,55	0,04	0,03	0,07	0,13	0,24	0,016
Needle 80 degrees	0,36	0,19	0,55	0,05	0,03	0,08	0,12	0,24	0,016
Needle 90 degrees	0,35	0,19	0,54	0,04	0,04	0,08	0,13	0,25	0,016