



Self-assembling silk for cell and drug delivery

Thesis presented by

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Declaration

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Abstract

In the past decade, silk has emerged as a promising biomaterial for biomedical and pharmaceutical applications, ranging from biomedical textiles to bioinspired drug-delivery nanoparticles with enhanced functionality. Although silk is widely regarded as a sustainable material, the translation of silk nanoparticles to industrial-scale applications remains challenging due to inconsistent production yields, scale-up limitations in manufacturing, and drug loading and delivery inefficiencies. Notably, silkworms possess a remarkable ability to spin silk fibers under physiological conditions by precisely regulating silk structural and conformational properties through various intrinsic and extrinsic factors, particularly metal ions. Thus, the central hypothesis of this thesis is that biomimicking the silkworm spinning mechanism may provide valuable insights into optimizing processing parameters to produce high-performance silk biomaterials while minimizing material waste during laboratory-scale silk nanoparticle manufacture.

To test this hypothesis, silk nanoparticles were manufactured using the antisolvent precipitation method in semi-batch format. Calcium ions (Ca^{2+}), a major ion present in the silk gland, were initially selected to optimize nanoparticle functionality and improve production yield. Spiking liquid silk with Ca^{2+} enhanced silk-silk interaction and self-assembly in a semi-batch format, resulting in a tunable nanoparticle size along with increased production yield. This optimized process also ultimately produced high biocompatible silk nanoparticles, as the resulting silk nanoparticles showed no toxicity and immunogenicity on the immune cell model, a RAW 264.7 murine macrophage (**Chapter 2**) (Roamcharern, N., *et al.*, ACS Biomater Sci Eng, **2025**, 11(3), 1847-1856).

To expand the understanding of metal ion modulation and its impact on silk nanoparticle fabrication *via* the antisolvent precipitation method, potassium ions (K^+) were investigated in parallel with calcium ions (Ca^{2+}). It was hypothesized that monovalent and divalent ions exert distinct effects on silk-silk interactions and/or self-assembly during nanoparticle formation, thereby yielding silk nanoparticles with distinct physicochemical properties. The findings demonstrated that K^+ and Ca^{2+} exerted coordinated effects on particle size, shape, and loading efficiency (Thioflavin T encapsulation), suggesting complementary underlying mechanisms. However, Ca^{2+} showed a greater impact on aqueous silk fibroin behavior than that of K^+ , as evidenced by the Thioflavin T assay and NMR analysis, resulting

in larger particle size and higher yield (**Chapter 3**) (Roamcharern, N., *et al.*, ACS Applied Bio Materials, **2025**, 8(8), 6854-6864).

The physicochemical stability of nanoparticles typically limits their therapeutic applications, as loss of physical stability, particularly colloidal stability, can negatively affect biologically relevant properties *in vivo*, thereby triggering immunogenic responses and reducing circulation time. This chapter hypothesizes that the incorporation of counterions improves the physicochemical stability of silk nanoparticles. To test this hypothesis, both short-term (2 h) and long-term (56 days) stability studies were conducted under various dispersants, pH conditions, and temperatures. Silk nanoparticles were characterized using complementary analytical techniques to assess their physicochemical stability, including DLS, ELS, NTA, SEM, AF4, and EAF4. The findings revealed that Ca^{2+} and K^{+} enhance the colloidal stability of silk nanoparticles during storage, particularly under elevated temperature and ionic stress conditions, thereby demonstrating their potential for drug delivery applications (**Chapter 4**) (Roamcharern, N., *et al.*, Nanoscale Adv, **2025**, 7(18), 5519-5535).

To enhance the suitability of silk nanoparticles for drug delivery, a Ca^{2+} - and K^{+} -free formulation was selected as a prototype for doxorubicin incorporation. Drug-loading efficiency was compared between direct antisolvent precipitation and post-fabrication adsorption, and release kinetics were evaluated under physiological, tumor microenvironment, and lysosomal pH conditions. The results demonstrated that the encapsulation method significantly influences physicochemical properties and pH-responsive drug release, with the adsorption-based formulation showing greater *in vitro* release. Moreover, compared to free doxorubicin, drug-loaded silk nanoparticles exhibited improved biocompatibility toward RAW 264.7 murine macrophages and enhanced selectivity against MDA-MB-231 and DU145 cancer cells. Collectively, these findings support the rational design of tunable silk-based nanocarriers for targeted doxorubicin delivery and establish a versatile manufacturing pipeline applicable to Ca^{2+} - and K^{+} -mediated silk nanoparticles (**Chapter 5**).

Overall, this thesis established the roles of Ca^{2+} and K^{+} in silk nanoparticle fabrication *via* the antisolvent precipitation method, along with a physicochemical and systematic evaluation pipeline using orthogonal analytical techniques (**Chapter 6**).

Research Outputs

Publications

- i. Punabhum, P.; Daramy, K.; **Roamcharern, N.**; Minelli, C.; Pei, Y.; Laleni, N. C.; Perrie, Y.; Rattray, Z. Analysis of bio-nano interactions by Electrical Asymmetric Flow Field-Flow Fractionation with multiple online detectors. *Journal of Chromatography A*, **2026**, 466879. DOI: <https://doi.org/10.1016/j.chroma.2026.466879>. (Journal IF: 4.0, Citation 0)
- ii. **Roamcharern, N.**; Punabhum, P.; Seib, F. P.; Rattray, Z. Evaluating the impact of bioinspired counterion inclusion on silk nanoparticle physicochemical attributes and physical stability, *Nanoscale Advances*, **2025**, 7(18), 5519-5535. DOI: <https://doi.org/10.1039/D5NA00365B>. (Journal IF: 4.6, Citation: 0)
- iii. **Roamcharern, N.**; Brady, D. J.; Parkinson, J. A.; Rattray, Z.; Seib, F. P. Optimizing Silk Nanoparticle Assembly with Potassium Ions: Effects on Physicochemical Properties and Encapsulation Efficiency, *ACS Applied Bio Materials*. **2025**, 8(8), 6854-6864. DOI: <https://doi.org/10.1021/acsabm.5c00598>. (Journal: 4.7, Citation: 2)
- iv. **Roamcharern, N.**; Matthew, S. A. L.; Brady, D. J.; Parkinson, J. A.; Rattray, Z.; Seib, F. P. Biomimetic silk nanoparticle manufacture: calcium ion-mediated assembly, *ACS biomaterials science & engineering*. **2025**, 11(3), 1847-1856. DOI: <https://doi.org/10.1021/acsbiomaterials.4c02175>. (Journal IF: 5.5, Citation: 10)
- v. Kaewchuchuen, J.; **Roamcharern, N.**; Phuagkhaopong, S.; Bimbo, L. M.; Seib, F. P. Microfibre-functionalised silk hydrogels. *Cells*. 2023, 13(1), 10. DOI: <https://doi.org/10.3390/cells13010010>. (Journal IF: 6, Citation: 4)

Conference contributions

Abstracts for conference contributions are listed in Appendices A–F.

- A. **Roamcharern, N.**; Punnabhum, P.; Seib, F. P.; Rattray, Z. (2025) ‘Modulation of Silk Nanoparticle Physicochemical Properties Through Calcium Ion Incorporation’. Poster presentation at **SIPBS Research Day 2025** 10 October 2025, Glasgow, United Kingdom.
- B. **Roamcharern, N.**; Seib, F. P.; Rattray, Z. (2025) ‘Ion-Mediated Modulation of Doxorubicin Encapsulation and Physicochemical Properties of Silk Nanoparticles: Role of Calcium and Potassium Ions Interaction’. Oral presentation at **16th APS PharmSci 2025** 9–11 September 2025, Cardiff, United Kingdom.
- C. **Roamcharern, N.**; Punnabhum, P.; Seib, F. P.; Rattray, Z. (2025) ‘Modulation of Silk Nanoparticle Physicochemical Properties through Calcium Ion Incorporation’. Poster presentation at **Samaggi Academic Conference and Careers Fair (SACC) 2025** 1 February 2025, London, United Kingdom.
- D. **Roamcharern, N.**; Seib, F. P.; Rattray, Z. (2024) ‘Modulating Silk Nanoparticle Size in Semi-Batch Format’. Poster presentation at **Malvern Panalytical’s 3rd Annual Pharma Conference** 11–12 June 2024, London, United Kingdom.
- E. **Roamcharern, N.**; Seib, F. P.; Rattray, Z. (2024) ‘Coordinating Silk Nanoparticle Properties’. Poster presentation at **15th APS PharmSci 2024** 4–6 September 2024, Huddersfield, United Kingdom.
- F. **Roamcharern, N.**; Matthew, S. A. L.; Rattray, Z.; Seib, F. P. (2023) ‘Improved Semi-batch Silk Fibroin Nanoparticle Manufacture’. Poster presentation at **14th APS PharmSci 2023** 5–7 September 2023, Reading, United Kingdom.

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List of Abbreviations

Abbreviation	Explanation
Å	Angstrom
Ala	Alanine
ANOVA	Analysis of Variance
°C	Degree Celsius
cm	Centimeter
cm ⁻¹	Reciprocal centimeter (unit of wavelength)
cm ²	Square centimeter
CO ₂	Carbon dioxide
cP	Centipoise
d.nm	Mean diameter of particle (unit of Z-average)
g	Gram
<i>g</i>	G-force
Gly	Glycine
GPa	Gigapascal
h	Hour
IR	Infrared
J kg ⁻¹	Joule per kilogram
kcps	Kilo counts per second
kDa	Kilodalton
kV	Kilovolt
L	Liter
M	Molar
mA	Milliampere
mb	Millibar
MHz	Megahertz
Min	Minute
mL	Milliliter
mm	Milliliter

Abbreviation	Explanation
mM	Millimolar
mg	Milligram
ms	Millisecond
mV	Millivolt
ng	Nanogram
nm	Nanometer
nM	Nanomolar
oscil/min	Oscillations per minute
pI	Isoelectric point
ppm	Part per million
rpm	Round per minute
Ser	Serine
SD	Standard deviation
Tyr	Tyrosine
Val	Valine
U/mL	Unit per milliliter
μL	Microliter
μm	Micrometer
μM	Micromolar
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight

Chapter 1 Introduction

1.1. Silk as a Biomedical Material

1.1.1. Silk Fibroin Protein Structure

Bombyx mori (*B. mori*) silk is recognized as the most common type of silk, emerging as a prominent subject of study in various research fields, from biomedical textiles¹ to nanoscale biomaterial therapeutics, especially nanoparticles,² due to its biocompatibility³⁻⁵ and biodegradability,^{6, 7} as well as its tensile strength and toughness.⁸ The high tensile strength and toughness of silk are key to its exceptional mechanical performance, and both are directly related to its protein structure, particularly the arrangement and interactions of its amino acid sequences.⁸

Silk fibroin (SF) is synthesized and secreted in the lumen of the posterior silk gland of a silkworm as a large protein complex consisting of three main components: (1) a high-molecular-weight heavy chain (H-chain, 390 kDa), (2) a low-molecular-weight light chain (L-chain, 26 kDa), and (3) a glycoprotein called fibrohexamerin (P25, 30 kDa), occurring at a 6:6:1 molar ratio.⁹ P25 is bound with the H-chain *via* a hydrogen bond; meanwhile, a disulfide bond links the H-chain and the L-chain. The H-chain occupies most of the SF and features both amorphous structures (β -turns, α -helices, and random coils) and crystalline β -sheet regions, accounting for $\sim 33\%$ and 66% of its sequence, respectively, and its majority amino acid composition is Gly (46%), Ala (30%), Ser (12%), Tyr (5.3%), Val (1.8%).^{10, 11} The non-repetitive amino acid residues are present at both the N- and C-termini of the H-chain. The central hydrophobic domain of the H-chain is dominated by repeating glycine-X (GX) units, where X can be Ala (65%), Ser (23%), or Tyr (9%). These sequences are organized into three main motifs: (1) GAGAGS, (2) GAGAGY or GAGAGVGY, and (3) GAGAGSGAAS.

The H-chain structure includes 12 hydrophobic blocks, each separated by 11 hydrophilic amorphous segments. Each hydrophobic motif serves a unique structural role:

GAGAGS primarily forms crystalline regions and appears at the start of each motif; GAGAGY and GAGAGVGY contribute to semicrystalline areas; and GAGAGSGAAS functions as a disruptive, sheet-breaking sequence.²

While in the middle silk gland, SF will be coated by silk sericin (SS) protein before being spun out towards the anterior silk gland, forming a silk fiber. This suggests that SF is the most abundant natural protein expressed in the cocoon of silkworms. It forms a stabilized complex with SS, which coats around SF filaments by van Der Waals forces and hydrogen bonds. This coupling provides an adhesive force between SF filaments, resulting in a strong integrity of silk cocoons. During the spinning process, the silkworm secretes and modifies the SF to form a long single silk fiber (20–30 μm) consisting of two brins (10–20 μm), each of which is composed by many nanofibrils (20–30 nm). The unique physicochemical properties of SF, such as rearrangement and cross-linking ability, are believed to play an important role in nanofibril formulation (**Figure 1.1**).¹²

Comprehensive knowledge of the silk fibroin structure remains limited; however, extensive NMR¹³ and related techniques, such as X-ray diffraction,¹⁴ IR,¹⁵ and Raman spectroscopy,¹⁶ have been widely used to validate its structure. According to recent understanding of SF structure, silk presents in two crystalline forms: silk I and silk II. These structures occur naturally in storage and spinning states,¹⁷ exhibiting different secondary structure content and rearrangements.¹⁸

Storage silk in the middle silk gland is preserved as a gel-like liquid state, which is believed to present as a silk I crystalline form.¹⁹ While silk I is often described as a random coil, ¹³C NMR studies of liquid *B. mori* silk from the fifth larval stage reveal that this simple random coil model insufficiently explains the transition to the stronger β -sheet-rich silk fiber formed during spinning.²⁰ The NMR profile of peptide (AG)₁₅, a silk amino acid model, indicates that type II β -turns are key structure in silk I, facilitating lamella (silk II) formation

through repetitive folding every eight residues. This is suggested to support structure conversion from silk I to silk II, predominantly governed by β -turns.^{20, 21}

The rearrangement of silk conformation from silk I to silk II, studied by NMR, revealed conformational transition, in which the chemical shift of key amino acids located in the GX repeat (e.g., Gly, Ala, and Ser) is altered *via* conversion to β -sheet conformation.²² A higher degree of β -sheet content is indicative of a silk II conformation, while lower β -sheet content and elevated random coil and β -turn content is indicative of a silk I conformation.²³ The factors that influence the silk conformational transition are further discussed in this thesis to clarify the mechanisms modulating this process.

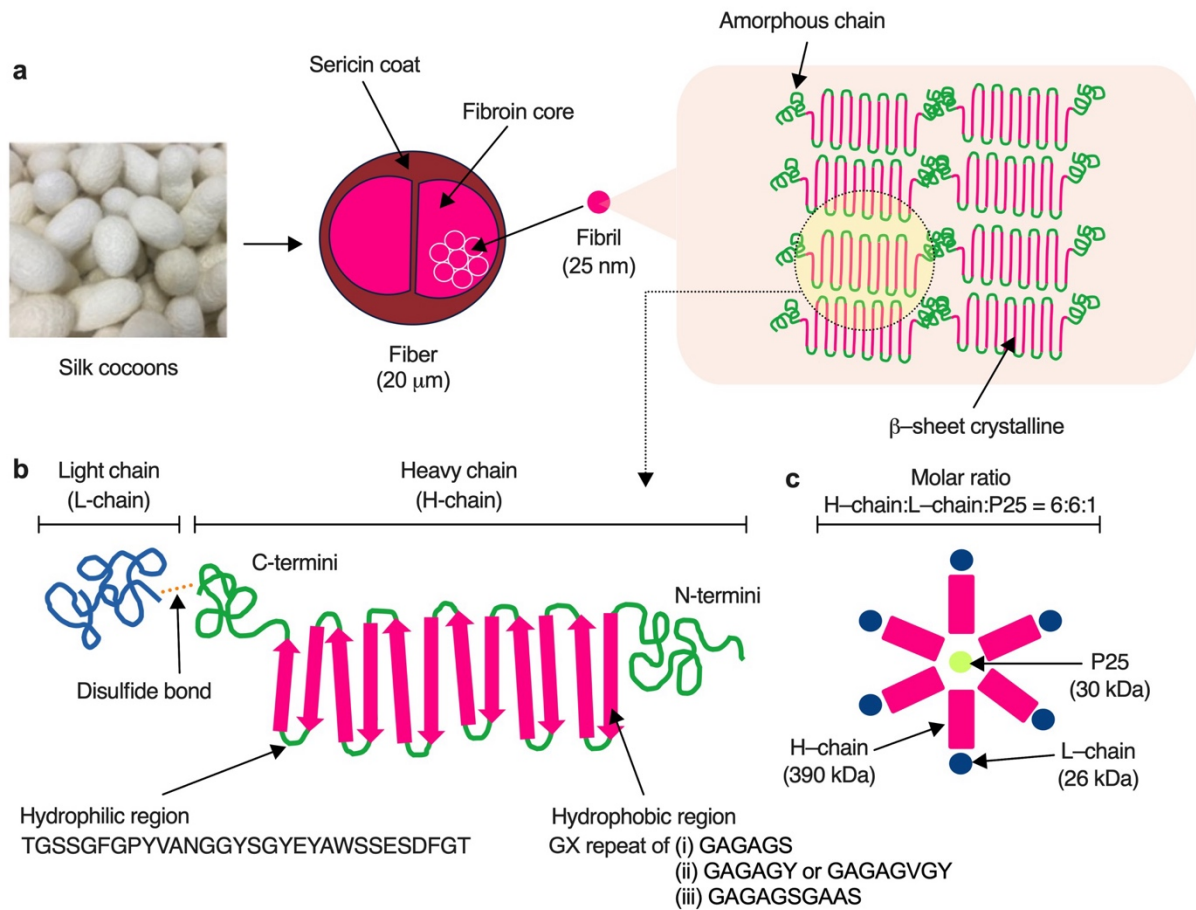


Figure 1.1 Silk fibroin protein structure: (a) *Bombyx mori* silk cocoons and the structure of fibril, (b) silk heavy chain and light chain, and (c) molar ratio of silk heavy chain, light chain, and fibrohexamerin (P25), adapted from^{24, 25}

1.1.2. Modulators of Silk Conformational Transitions

The remarkable ability of the silkworm to spin silk fibers under physiological conditions—at ambient temperature, in the absence of elevated hydrostatic pressure or toxic chemicals—is considered a key model for *ex vivo* silk material processing.²⁶ During silk fiber spinning, silk polymer assembly is driven by water extraction, gradual shifts in pH,²⁷ changes in metal ion concentrations,²⁸ and shear force,²⁹ which together promote the alignment of silk polymer chains- the conformational transition from silk I to silk II.²⁶ Therefore, a clear understanding of this regulatory mechanism may provide valuable insights for optimizing the silk tuning process to generate high-performance silk biomaterials.

The silkworm has an important organ used in silk synthesis and storage. This organ can be stratified into three main sections: the posterior (P), middle (M), and anterior (A) silk gland. The wide section of the middle silk gland is further categorized as posterior (MP), middle (MM), and anterior (MA),^{27, 30} which occupy different functions in silk management: (1) silk fibroin protein synthesis occurs in the posterior silk gland, (2) sericin protein addition and silk storage occur in the middle silk gland, expressing a high concentrated silk at ~20–25% *w/w*,³⁰ and (3) silk secretion passing through the narrow anterior silk gland towards spinneret during silk fiber spinning.¹⁷

In the silk gland, pH and viscosity decrease from the posterior to the anterior compartments,²⁷ while the gradient of metal ions varies.²⁸ Among the six reported elements, including Na⁺, K⁺, Mg²⁺, Ca²⁺, Cu²⁺, and Zn²⁺, Ca²⁺ and K⁺ are reported as the most abundant elements in the middle silk gland, where the silk fibroin is preserved,²⁸ and exhibit the strongest impact on silk structural modulation.³¹ Interestingly, a decrease in Ca²⁺ level is observed, varying in the posterior and anterior divisions of the middle silk gland, lowering from ~ 2,500–500 µg/g dry weight in luminal contents. In contrast, K⁺ levels rise from ~ 3,000–11,000 µg/g dry weight in luminal contents.²⁸

Typically, the silk conformational transition is modulated by the synergistic effects of pH, metal ions, and shear force. In the anterior silk gland, a reduction in pH from pH 6.9 to pH 4.8 occurs towards the spinneret.^{27, 32} pH plays a critical role in β -sheet formation, stabilization, and the regulation of the liquid-crystalline transition. This process is governed by the ionization of acidic amino acids on the silk H-chain. A high concentration of these acidic amino acids is found at the N-terminus and within the hydrophilic spacers between large hydrophobic domains. The theoretical isoelectric point (pI) for silk H-chain is 4.39;³³ thus, at a near-physiological pH of 6.9 (pH in the middle silk gland lumen), a substantial negative charge predominates, resulting in strong repulsive forces between protein molecules. When the pH decreases to 4.8 (spinning duct pH), the ionization level drops, creating a better balance between repulsive and attractive forces. This shift facilitates the stabilization of hydrophobic domains by bringing them closer and maintains the nematic liquid-crystalline state in specific regions of the spinning duct. Together with increased viscosity, rapid extensional flow, and exposure to air and ambient conditions, dehydration occurs, ultimately leading to fiber formation.³²

It is important to note that silk conformational transition is modulated not only by pH but also by changes in metal ion concentrations along the secretory pathway. The Ca^{2+} levels increase between the posterior and middle silk gland, while a drop in Ca^{2+} is observed in the middle anterior silk gland (proximal spinning duct), leading to a gel-sol transition with lower viscosity.²⁶ Physiological levels of calcium ions promote the formation of β -sheets and the distorted β -sheet structures characteristic of silk II, whereas elevated calcium concentrations preserve the random coil conformation (storage silk I).³⁴ Furthermore, Ca^{2+} is hypothesized to be involved in β -sheet formation, nucleation and/or stabilization of the crystalline microstructure of silk fiber.²⁶ As the concentration of K^+ increases toward the anterior silk

gland, K^+ interacts with silk fibroin through electrostatic interactions, inducing the conversion of random coil structures into β -sheets.³⁵

The Ca^{2+} (ionic radius = 0.99 Å) and K^+ (ionic radius = 1.33 Å) cations exhibit different impacts on silk fluid characteristics due to their different coordination number, with Ca^{2+} having 10–12-fold more coordination sites than K^+ during interaction with silk fibroin. This results in more binding sites per fibroin chain, resulting in stronger interactions that limit the mobility of silk fibroin in solution and yield a highly concentrated viscous silk feedstock in the middle silk gland.³⁶

In addition to the transient salt bridges formed between metal ions and negatively charged amino acid residues (e.g., aspartate and glutamate) in hydrophilic spacers and the N-terminus of the silk H-chain,²⁸ the formation of coordination complexes between metal ions and amino acid residues in hydrophobic regions (histidine,²⁸ serine,¹⁴ and tyrosine)¹⁵ has also been strongly implicated in silk β -sheet formation. Taken together, the role of metal ions and pH during silk fiber spinning is critical. The transition from silk I to silk II, is characterized by a decrease in Ca^{2+} levels, an increase in K^+ levels, and a reduction in pH (**Figure 1.2**).

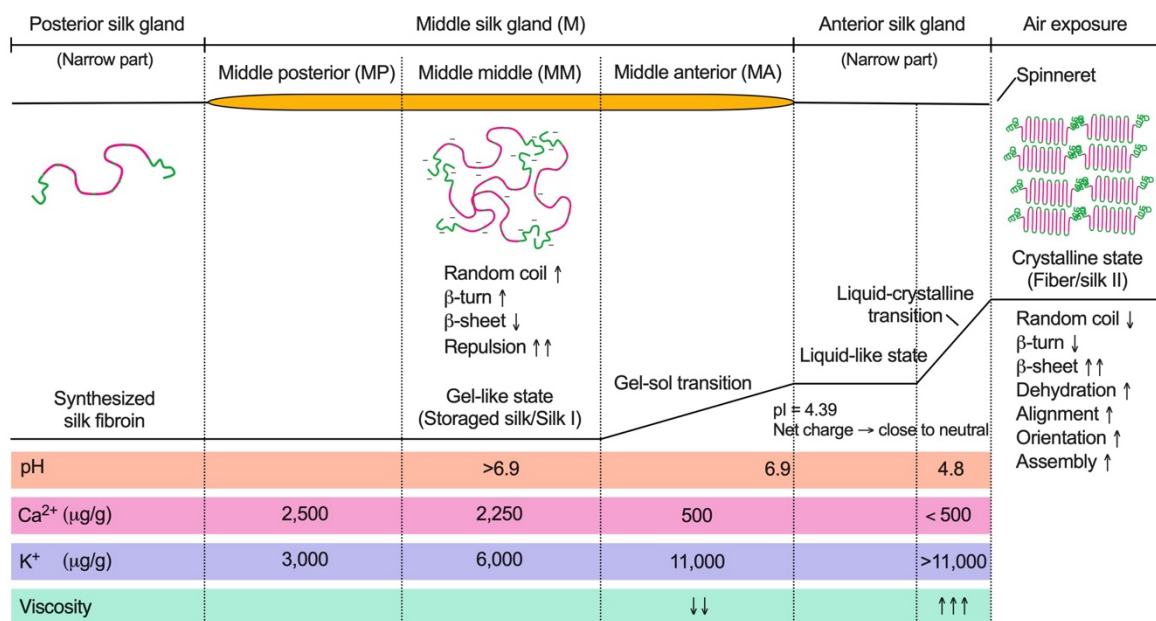


Figure 1.2 The proposed mechanism for modulating the conformational transition of silk fibroin during fiber spinning in the silkworm. The transition of silk I to silk II involves the synergistic effects of pH, metal ions (Ca²⁺ and K⁺), and viscosity.^{16, 18, 26, 27, 30, 32-38}

1.1.3. Silk Tensile Strength and Toughness

The semicrystalline structure of silk fibroin is facilitated by hydrophobic amino acids, particularly glycine, alanine, and serine, which contribute to its tensile strength and toughness. Typically, the commercial silkworm silk possesses a tensile strength of approximately 0.5 GPa and a toughness (breaking energy) of $6 \times 10^4 \text{ J kg}^{-1}$;⁸ however, these mechanical properties of silk are influenced by various factors, such as silkworm instar stages³⁹ its diet,⁴⁰ environmental conditions (e.g., temperature and humidity),⁴¹ and reeling speed of silk fibre,⁴² primarily modulating the degree of crystallinity.

Re-engineered silk is produced through various physical and chemical treatments of silk cocoons, as described in point, including the essential step used in sericin removal, known as degumming. The degumming process is an excellent example of an initial and important factor in optimizing the tensile strength and toughness of silk in *ex vivo* applications, enabling changes in the microstructure of the fibroin core.^{43, 44} Therefore, understanding the fundamental

properties of silk has led to promising methods for tuning its mechanical strength and toughness, key factors for designing versatile silk-based biomaterials.

1.1.4. Silk Biodegradation and Biocompatibility

The biodegradation behaviour of silk depends on its molecular weight and structure. A lower molecular weight and a less compact structure facilitate a stronger interaction between the enzyme and silk, thereby enhancing hydrolysis activity.⁴⁵ The correlation between silk structure and its biodegradation has also been reported elsewhere, describing that the protease XVI easily performs a surface erosion mechanism through the pore surfaces, leading to particle fragmentation, particle size reduction, and 60% mass loss by 12 days.⁴⁶ In addition, protease XVI tends to hydrolyze the hydrophilic segments, an amorphous region, more readily than the stacked β -sheet and/or tightly packed β -turn aggregates.⁴⁷ Taken together, the degradation behavior of silk biomaterial is governed by several factors, including (1) secondary structure composition and content, especially the β -sheet content,⁴⁸ (2) silk biomaterial format,⁴⁶ (3) proteolytic enzyme systems and biodegradation condition (e.g., enzyme types, enzyme-to-silk ratio, exposure time, temperature, pH).⁴⁹

In addition to its biodegradability, silk biocompatibility has been widely recognized in medical and pharmaceutical applications, establishing it as a versatile biomaterial. For centuries, silk has been valued for its remarkable properties—including ease of processing, controllable degradability, favorable mechanical strength, and excellent biocompatibility.¹ These characteristics have made silk a widely accepted biomedical textile and have enabled its exploration in advanced areas of biomedical engineering, ranging from implantable materials⁵⁰ to non-implantable medical devices.⁵¹ Silk scaffolds are highly biocompatible and well-suited for tissue engineering. Silk fibers have long been used as medical sutures, valued for their strength and biocompatibility. Ongoing research aims to enhance these properties for diverse wound healing applications.⁵² Silk biomaterials have been explored in tissue artificial implant

technology, showing their potential for use in tissue engineering approaches. (e.g., neurosurgery,^{4, 53} bone and cartilage engineering,⁵⁰ vascular engineering,^{54, 55} skin tissue engineering,⁵⁶ and gel/film/microsphere from silk⁵⁷).

1.2. Bioinspired Silk Biomaterial: Silk Nanoparticles for Advanced Drug Delivery

1.2.1. Reverse-Engineered Silk

Recently, the silkworm silk has emerged as a critical protein used in medical and pharmaceutical approaches. The domesticated *B. mori* silk, also referred to as “mulberry silk”- named after its natural diet- has gained extensive attention in the silk industry worldwide.⁵⁸ Apart from properties as an ideal biomaterial, the silkworm can be easily farmed (sericulture) for multiple purposes, such as future protein-based food (edible silkworm)⁵⁹ and sustainable and economical textile industry (spun silk cocoons)⁶⁰ with a favorable silk production yield (350 mg per cocoon)⁵⁸ and suitability for downstream extraction protocols.

The processing of silk for reverse-engineered silk production has been the subject of extensive study. By taking advantage of its structure and hierarchical assembly (sections 1.1.1 and 1.1.2), numerous fabrication strategies have emerged that enable the tailoring of silk structure at the nanoscale, offering broad opportunities to convert silk cocoons into various formats, including fibers,⁶¹ nanofibers,⁶² films,⁶² powder,⁶³ hydrogels,⁶⁴ and nanoparticles.²³ Generally, reverse-engineered silk solution (liquid silk) is generated as a starting material for almost all fabrication of those silk formats, except for the raw silk fibers in which the production of regenerated silk may not be required.⁴² Production of liquid silk involves several chemical and physical treatments, as categorized into (1) degumming, (2) dissolution, and (3) purification (**Figure 1.3**).

(1) The first step of liquid silk preparation is called the degumming step, sometimes referred to as sericin removal.⁶⁵ The chopped cocoons are boiled in a degumming liquor, such

as the most widely used aqueous solution of sodium carbonate (Na_2CO_3)²³ (**Table 1.1**), under heat. During the degumming process, the extraction of sericin, a glue-like protein coating on the surface of twin silk fibroin monofilaments, occurs due to the hydrolysis of its peptide bonds induced by Na_2CO_3 —which generates alkaline conditions, enhancing sericin solubility. Meanwhile, silk fibroin polypeptide chains possess greater physical stability, reinforced by both inter- and intra-molecular bonding that stabilize their specific β -sheet folding and conformation. Therefore, during the degumming process, silk retains its strength and toughness, presenting as an insoluble degummed raw silk (cotton-like form).⁶⁶ However, harsh degumming conditions impact not only the remaining sericin content but also the performance and qualities of degummed silk (e.g., damage, degradation, yield, and physicochemical properties) as evidenced by the presence of several impairments as increased degumming effects or stronger conditions (e.g., increased Na_2CO_3 concentration, elevated temperature, and prolonged boiling time).⁶⁷ In addition, the degumming conditions can influence the cytotoxicity and reproducibility of silk, raising significant concerns for its future medical applications (**Figure 1.3a**).⁶⁸

(2) Dissolution is the process of dissolving degummed silk in the presence of a dissolving solvent. The mechanism of action for this process is that the dissolving solvent initiates the disruption of the inter- and intra-molecular bonding, mainly the hydrogen bonding and van der Waals forces, resulting in destabilization of silk β -sheet crystallinity.⁶⁶ Therefore, various high-ionic-strength aqueous solutions of organic salts have been explored for their ability to facilitate liquid silk generation. For example, Ajisawa's reagent⁶⁹ and lithium salt solution system (e.g. lithium thiocyanate (LiSCN)⁷⁰ and lithium bromide (LiBr)).²³ Both Ajisawa's reagent and lithium salts are the most frequently used dissolving solvent systems for reverse-engineered silk production, addressing it as a fast, convenient, and reliable method.⁷¹

Using different solvents for dissolution, regenerated silk with varying physicochemical characteristics can be obtained. The LiBr solution enables effective dissolution of degummed silk by promoting multilevel structural degradation, generating low-molecular-weight products, and reducing crystallinity. This, in turn, yields a structure rich in random coils and β -turns, which is beneficial for studying conformational transitions.⁷² Similar benefits can be obtained with LiSCN. However, its toxicity is more likely to limit its application in biomedicine.⁷² Beyond solvent systems for silk dissolution, key factors impacting silk dissolution efficiency include temperature, dissolution time, and stirring speed (**Figure 1.3b**).⁶⁶

(3) The purification process is carried out after the dissolution step, aiming to remove all residual solvents and contaminants from the reverse-engineered silk solution. Due to the widespread use of salt system dissolution (e.g., lithium salt and Ajisawa's ternary solvent), dialysis has emerged as a common purification technique, owing to its advantages in salt removal and/or reduction from protein solutions.⁷³ Dialysis requires the target protein solution to be placed into dialysis tubing or a cassette, which contains a semi-permeable membrane enclosing the solution. The sealed tubing or cassette is then immersed in a larger container filled with water or the desired exchange buffer. Salt removal relies on passive diffusion based on concentration gradients and is also dependent on the molecular weight cut-off (MWCO) of the dialysis membrane. Salt and/or impurities that possess a smaller hydrodynamic radius (R_h) can diffuse through a membrane, while target proteins with a higher hydrodynamic radius than the membrane pores, are retained by the membrane.⁷³ Purification by dialysis is time-dependent, depending on the diffusion kinetics regulated by several parameters such as starting concentration, solvent volume, and solvent exchange method (e.g., continuous flow or one-time exchange),⁷⁴ and equilibrium is reached when the final salt concentration in the protein solution is equal to the final salt concentration in the dialysate.⁷³ Therefore, the dialysate must be exchanged repeatedly during the process until the salt is completely removed (**Figure 1.3c**).

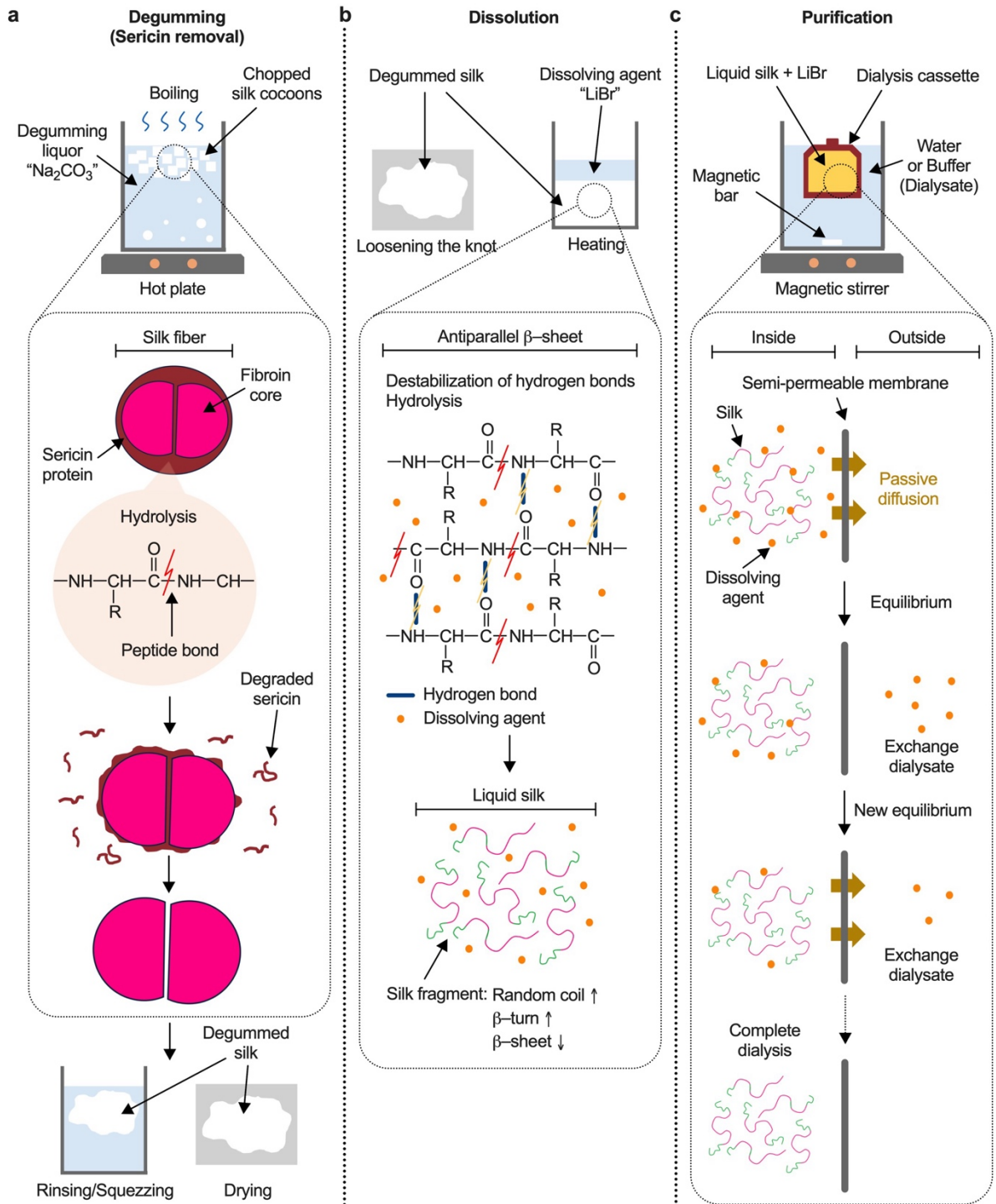


Figure 1.3 Schematic of generating the reverse-engineered aqueous silk fibroin: (a) degumming, (b) dissolution, and (c) purification.^{65, 67, 72, 73}

1.2.2. Manufacture of Silk Nanoparticles

1.2.2.1. Antisolvent Precipitation Method

It is well-established that the strategies used in nanoparticle production can be classified as (1) top-down: breaking down bulk materials or large particles into nanoparticles, and (2) bottom-up: assembling small components/molecules to form nanoparticles. Notably, bottom-up approaches require less energy in processing than top-down methods,⁷⁵ thereby facilitating the large-scale production of protein-based nanoparticles, including emulsion,⁷⁶ complex coacervation,⁷⁷ nano spray drying,⁷⁸ electrospraying,⁷⁹ self-assembly,⁸⁰ and antisolvent precipitation.^{23, 81, 82}

Several techniques have been developed for silk nanoparticle manufacture from liquid fibroin precursor, with antisolvent precipitation confirmed as a highly effective method due to its operational simplicity, lack of requirement for expensive equipment, favorable time efficiency, excellent reproducibility,² and offering fine-tuned nanoparticles with controlled physicochemical properties.⁷⁵

Antisolvent precipitation, sometimes referred to as desolvation,⁷⁵ involves three fundamental steps: supersaturation, nucleation, and particle growth within the combined solvent-antisolvent system. This process begins by (1) solubilizing the biopolymer in a solvent, followed by the controlled introduction of an antisolvent in which the biopolymer has low solubility. During the continuous addition of the solute to the antisolvent, at a certain time point, when the solute concentration exceeds the critical saturation concentration, “supersaturation” occurs. Next, (2) interactions between the solute, solvent, and antisolvent become imbalanced, the solvent-antisolvent environment eventually favors solute-solute interactions strong enough to overcome the energy barrier (ΔG) of mixing, resulting in “nucleation”. Higher supersaturation and lower surface tension of the solute reduce the critical ΔG , resulting in a faster nucleation rate. This, in turn, leads to a uniform distribution of nuclei and limited particle

growth, yielding a narrow particle size distribution. Finally, (3) a lower concentration of solute occurs during the nucleation process, leading to particle growth by condensation and agglomeration. Condensation refers to the adsorption of the free solutes onto the particle surface, enlarging the particle size after nucleation. However, once the solute concentration drops below the equilibrium saturation concentration, the condensation-induced particle growth stops. Beyond this point, particle growth is governed mostly by agglomeration, which involves particle collision frequency/efficiency and particle-particle interactions (e.g., van der Waals force and electrostatic repulsion) (**Figure 1.4**).⁷⁵

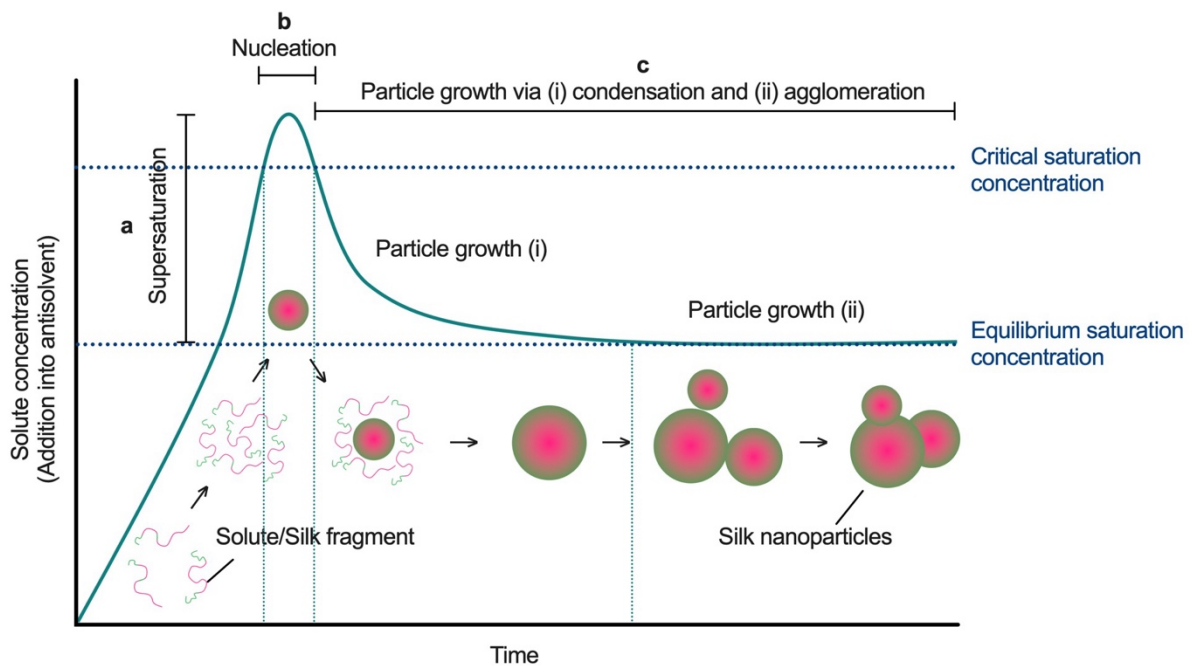


Figure 1.4 The mechanism of nanoparticle formation via antisolvent precipitation. The silk nanoparticle formation process includes (a) supersaturation, (b) nucleation, and (c) particle growth *via* (i) condensation and (ii) agglomeration, adapted from⁷⁵

1.2.2.2. Factors Influencing Nanoparticle Formation During Antisolvent Precipitation

A solid understanding of particle size control during antisolvent precipitation is essential for the effective design of mixing systems.⁸³ The triggers influencing particle formation during antisolvent precipitation are both chemical and physical factors. The chemical factors include: (1) the solvent-to-antisolvent ratio, which dictates silk solubility and behavior within the mixed system;⁸⁴ (2) the presence and concentration of surfactants and/or stabilizers, which impact nucleation and growth through interfacial tension, thereby enabling particle size control and improving stability and polydispersity;^{75, 85, 86} (3) the reaction pH, which influences particle size, polydispersity, and particle morphology;⁸⁷ and (4) temperature, where elevated conditions can promote particle growth by lowering a supersaturation, enhancing molecular diffusion and collision frequency, and weakening solvation effects.⁸⁸ Physical factors including feed rate, stirring speed, and aging time further affect supersaturation, dispersion, and crystallinity during antisolvent precipitation.⁸⁴

Recently, aqueous silk has drawn increasing attention as a starting material for silk nanoparticle fabrication.²³ Similar to other protein-based nanoparticles, silk nanoparticles can be produced using various bottom-up methods.

In the antisolvent precipitation system, physical parameters such as stirring rate (0–400 rpm) and initial dropping height (5.5 vs. 7.5 cm) significantly affected particle size and yield, as evidenced by isopropanol-mediated drop-by-drop precipitation.²³ In contrast, standing time (0–24 h) had minimal impact on agglomeration and aggregation, as a result of repulsive electrostatic interactions between silk nanoparticles and silk chains, indicating high colloidal stability. The system showed good scalability and reproducibility with a six-fold volume increase. However, these parameters did not reduce residual silk loss, which remained high (~80%) during precipitation.²³ The effects of batch volume and mixing time are also addressed as key factors influencing the physicochemical properties, crystallinity, and reproducibility of

silk nanoparticles.⁸⁹ In addition, mixing and flow-induced precipitation were identified as modulators of morphological optimization, relying on the shear rate during the precipitation process. A shear rate below the silk critical shear threshold preserved particle sphericity, reduced particle size, and the polydispersity index.⁹⁰

Silk nanoparticles with high drug encapsulation efficiency are promising for drug delivery. Antisolvent precipitation enables precise control of their properties, with the blending-to-silk ratio notably influencing size, polydispersity, and surface charge.⁹¹ To reduce silk nanoparticle agglomeration and tailor their properties, surfactant incorporation has been explored.⁹² Modifying the solvent-antisolvent environment, particularly by selecting optimal antisolvents, aids the fine-tuning of silk nanoparticle performance. Various inorganic solvents have proven effective in this process (**Table 1.1**).

This thesis addresses gaps in lab-scale antisolvent precipitation by exploring naturally-inspired chemical triggers—specifically Ca^{2+} and K^{+} ions—for modulating silk physicochemical properties (section 1.1.2). While ions like Ca^{2+} and K^{+} have shown promise in size tuning during self-assembly of *Antheraea pernyi* silk,¹⁸ their effects on *B. mori* silk nanoparticles *via* antisolvent precipitation remain largely unexplored.

Table 1.1 Bombyx mori silk nanoparticles generated by antisolvent precipitation.

Aim/Desired functionality	Degumming/ Dissolution agents	Solvent	Antisolvent	Parameters		Payload	Size/Morphology	Limitations	Reference
				Fixed	Optimized				
Establish a semi-batch silk nanoprecipitation	- 0.02 M Na ₂ CO ₃ (98–105°C, 60 min) - 9.3 M LiBr (60°C, 4 h)	Ultrapure water	Isopropanol	✓	- Dropping height - Stirring rate - Aging time	–	104–134 nm/Sphere	Significant silk wastage (~80%)	²³
Optimize volumetric scale up	- 0.02 M Na ₂ CO ₃ (98–105°C, 60 min) - 9.3 M LiBr (60°C, 4 h)	Ultrapure water	Isopropanol	✓	- Flow rate/Shear rate - Total volume - Mixing time	–	100–200 nm/Sphere	Significant silk wastage (~65%)	⁸⁹
Control the morphology	- 0.02 M Na ₂ CO ₃ (98–105°C, 60 min) - 9.3 M LiBr (60°C, 4 h)	Ultrapure water	Isopropanol	✓	- Silk concentration - Needle internal diameter - Dropping height - Flow rate/Shear rate - Stirring rate - Mixing time - Evaporation control	–	100–200 nm/Sphere, Rod-like, Nanofiber, Amorphous	Significant silk wastage (~65%)	⁹⁰
Establish a semi-batch silk nanoprecipitation	- 0.02 M Na ₂ CO ₃ (Boiling, 60 min) - 9.3 M LiBr (60°C, 4 h)	Ultrapure water	Acetone	✓	- Silk concentration - Drop-wised rate	Doxorubicin HCl (Adsorption)	107 nm/Sphere	No varying in parameter impacts	⁹³
Improve loading efficiency for natural compounds	- 0.05 M Na ₂ CO ₃ (Boiling, 2 h) - 9.3 M LiBr (65°C, 3 h)	Ultrapure water	Methanol	✓	- Stirring time (2 h more for suspension)	Mixed natural polyphenols (PPh) (Adsorption)	~170 nm/Sphere	Risk of LiBr contamination/ require >2 h for production	⁹⁴
Improve colloidal stability and yielding using stabilizing agents	- Na ₂ CO ₃ (Boiling, 30 min) - CaCl ₂ :Ethanol:H ₂ O (1:2:8, molar ratio) (20–30°C, 1 h)	Ultrapure water	Acetone	✓	- Surfactants/Stabilizing agents (e.g., Tween 80, Kollidon VA64, and Poloxamer 188) - Concentration of stabilizing agents	–	294–1655 nm/Sphere	No solid evidence on improved production yield	⁹²

Table 1.1 Bombyx mori silk nanoparticles generated by antisolvent precipitation.

Aim/Desired functionality	Degumming/ Dissolution agents	Solvent	Antisolvent	Parameters		Payload	Size/Morphology	Limitations	Reference
				Fixed	Optimized				
Produce zein-silk/sericin nanoparticle for transdermal route delivery	Commercial silk sericin protein (99%, Mw 40 kDa, Xi'an Baichuan Co. Ltd., China).	Ultrapure water	Ethanol	✓	- Blending (Zein protein) concentration	Curcumin	330–400 nm/Sphere	pH adjustment and high stirring rate requires	⁹¹
Produce a delivery vector for controlled release of payload	- 0.02 N Na ₂ CO ₃ (Boiling, 45 min) - 10% CaCl ₂ :Ethanol:H ₂ O (1:2:8, molar ratio) (70°C, 6 h)	Ultrapure water	Methanol	✓	- Stirring time (2 h more for suspension)	Resveratrol (Adsorption)	~100 nm/Sphere	Require >2 h for production/Temperature control	⁹⁵
Produce silk nanoparticle for osteoarthritis (OA) treatment	- 0.02 M Na ₂ CO ₃ (Boiling, 30 min) - 9.3 M LiBr (60°C, 4 h)	Ultrapure water	Acetone	✓	- Payload ratio	Celecoxib and curcumin	113–118 nm/Sphere	Dialysis and freeze-drying process require	⁹⁶
Improve colloidal stability of silk nanoparticle	- 0.5% Na ₂ CO ₃ (Boiling, 60 min) - CaCl ₂ :Ethanol:H ₂ O (1:2:8, molar ratio) (90°C, 2 h)	Ultrapure water	Acetone	✓	- Blending (cationic polymers) concentration - Blending type	Doxorubicin HCl (Adsorption)	168–201 nm/Sphere	Require additional stirring step for acetone removal	⁹⁷
Produce silk nanoparticle for severe bone infection treatment	- 0.02 M Na ₂ CO ₃ (Boiling, 30 min) - 9.3 M LiBr (60°C, 4 h)	Ultrapure water	Acetone	✓	- Silk concentration - Solvent/antisolvent volume ratio	Vancomycin	80–90 nm/Sphere	No varying in parameter impacts	⁹⁸
Promote a novel carrier-in-carrier delivery system	- 0.02 M Na ₂ CO ₃ (Boiling, 30 min) - 9.3 M LiBr (60°C, 4 h)	Ultrapure water	Acetone	✓	- Payload ratio	Curcumin	100 nm/Sphere	Dialysis and freeze-drying process require	⁹⁹
Produce silk nanoparticle for	- 0.5% Na ₂ CO ₃ (Boiling, 30 min, twice)	Ultrapure water	Ethanol	✓	- Silk concentration - Payload ratio	Paclitaxel	158–206 nm/Sphere	Require particle filtration	¹⁰⁰

Table 1.1 Bombyx mori silk nanoparticles generated by antisolvent precipitation.

Aim/Desired functionality	Degumming/ Dissolution agents	Solvent	Antisolvent	Parameters		Payload	Size/Morphology	Limitations	Reference
				Fixed	Optimized				
locoregional drug delivery	- CaCl ₂ :Ethanol:H ₂ O (1:2:8, molar ratio) (70°C, 4 h)		Methanol Acetone		- Antisolvent type				
Produce silk nanoparticle for drug delivery	- 0.02 M Na ₂ CO ₃ (Boiling, 60 min) - 9.3 M LiBr (60°C, 4 h)	Ultrapure water	DMSO	✓	- Silk concentration - Dropping rate - Stirring rate	–	150–170 nm/Sphere	Require particle filtration	¹⁰¹
Explore silk nanoparticle physical attributes formulated by optimizing dissolution time and organic solvents	- 0.5% Na ₂ CO ₃ (Boiling, 30 min) - CaCl ₂ :Ethanol:H ₂ O (1:2:8, molar ratio) (90°C, 2-12 h)	Ultrapure water	Methanol Ethanol Propanol Isopropanol Tetrahydrofuran Acetone Acetonitrile	✓	- Antisolvent type	–	35–125 nm/Sphere	Require particle filtration	¹⁰²
Produce silk nanoparticle for nitric oxide delivery	- 0.02 M Na ₂ CO ₃ (Boiling, 30 min) - 9.3 M LiBr (60°C, 4 h)	Ultrapure water	Ethanol	✓	- Silk concentration - Stirring rate	S-nitroso-N-acetylpenicillamine	300–400 nm/Sphere	Require freezing (–20°C) and thawing (RT) to form milky emulsion	¹⁰³

1.2.3. Analytical techniques employed in silk nanoparticle characterization

1.2.3.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful analytical techniques used in the study of the structure, dynamics, and chemical kinetics of biomolecules. The use of advanced NMR technology to determine the three-dimensional structure of proteins emerged in the early 1980s, initially enabling the study of proteins without requiring crystallographic data.¹⁰⁴

The fundamental principle of NMR spectroscopy involves the magnetic properties of nuclei, which absorb energy at a specific frequency of an applied magnetic field. The nucleus can behave like a microscopic magnet due to its nuclear spin. Consequently, the spin behaviour of electrons around neighbouring protons within a molecule can influence one another—this phenomenon is known as nuclear magnetic resonance. Differences in the chemical environment (electronic environment) of protons lead to varying degrees of electronic shielding, which in turn causes them to resonate at different frequencies when excited. NMR spectra are typically analysed based on three main characteristics: chemical shift, integration, and splitting. In the case of proton NMR spectra, the spectra can be easily plotted as a chemical shift scale, which is the relative frequency referenced by a standard compound, tetramethylsilane (TMS).^{105, 106} The proton, which is far from an electronegative element, will hold a higher electronic shielding with lower electronic spinning frequency, expressing an NMR signal toward an upfield position (lower ppm values), while those deshielding will be shifted towards a downfield position (higher ppm values).¹⁰⁵ Thus, the chemical shift can refer to the chemical environment of a proton in a compound. Importantly, protons that share the same chemical environment—for example, the three protons in the methyl group ($-\text{CH}_3$) of ethanol ($\text{CH}_3-\text{CH}_2-\text{OH}$)—have their signals integrated and appear at the same chemical shift as a single peak. However, the microscopic magnet of the neighbouring protons can influence

the adjacent protons through a J-coupling effect, resulting in a splitting of a signal. Therefore, the signal of a proton in $-\text{CH}_3$ will be split into a triplet not a singlet due to its neighbouring protons in the methylene group ($-\text{CH}_2$). The splitting pattern (multiplicity) can be calculated by $n+1$, where n is the number of neighbouring protons. Thus, the chemical shift of two protons in the $-\text{CH}_2$ group will also be split into a quadruplet.¹⁰⁵⁻¹⁰⁸

The structures of silkworm silk fibroin—specifically silk I and silk II—have been widely studied using NMR spectroscopy. The chemical shift patterns of major amino acid residues, including glycine,¹³ alanine,¹³ serine,^{13, 14} tyrosine,¹⁵ and histidine,¹⁶ observed in the ^1H , ^{13}C , and ^{15}N NMR spectra indicate conformation-dependent chemical shifts, highlighting the potential of NMR for the quantitative analysis of silk structure.

Metal ions—specifically Ca^{2+} and K^+ —have been reported as key factors regulating silk conformation and structure during storage and fiber spinning (section 1.1.2).^{18, 37, 109} NMR analysis revealed conformational changes in silk films prepared with different concentrations of K^+ (2.5–3.7 mg K^+ /g of silk), reflecting its influence on silk secondary structure transition: helix and/or random coil $\rightarrow$ helix-like $\rightarrow$ β -sheet-like $\rightarrow$ β -sheet. In addition, chemical shift changes of tyrosyl C_α were slightly shifted toward an upfield direction (from 57.5 to 55.5 ppm), indicating changes in hydrophobicity during the silk structure transition (helix $\rightarrow$ β -sheet). However, with a difference in ionic radii and coordination number between Ca^{2+} and K^+ , they could exhibit distinct effects on silk structural transitions, as evidenced by different interaction degrees of different metallic species with the carbonyl oxygen of tyrosine residues.^{15, 110}

Furthermore, due to limited understanding of the impact of Ca^{2+} and K^+ on regenerated silk fibroin and their size-tuning capabilities in antisolvent precipitation, it is necessary to investigate these interactions further. Therefore, this thesis includes an NMR study of liquid silk fibroin titrated with Ca^{2+} and K^+ before silk nanoparticle synthesis. This approach examines their roles through changes in chemical shift profiles, line shapes, and peak intensities in the

^1H and ^{15}N NMR spectra. Using NMR, provides insights into the solution phase behaviour of silk before undergoing a liquid-crystalline transition, demonstrating that the conformation and structure of silk can be modulated by metal ions, thereby paving the way for optimization of silk nanoparticle fabrication *via* antisolvent precipitation (**Figure 1.5**).

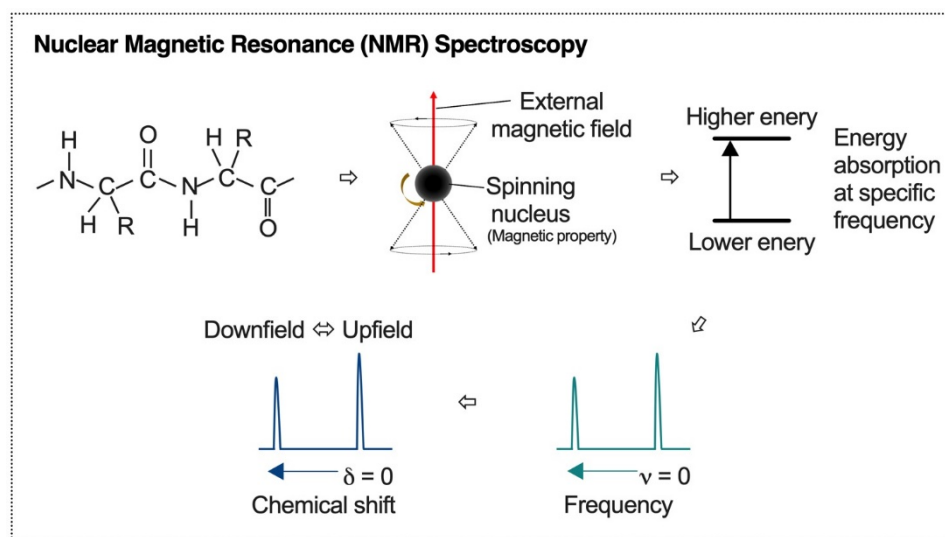


Figure 1.5 Schematic showing the principle of Nuclear Magnetic Resonance (NMR) Spectroscopy, adapted from¹¹¹

1.2.3.2. Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a non-destructive method for measuring the hydrodynamic size of suspended particles sized 1–1000 nm in diameter.¹¹² Typically, particles in suspension are illuminated with a laser beam, scattering light at various angles and directions.¹¹³ DLS detects and records the scattered light at 90° and 173° .¹¹⁴ DLS measures fluctuations in the intensity of scattered light over time, where the frequency of light scattered depends on the velocity of particles experiencing random movement in the liquid, also known as Brownian motion. Smaller particles move faster in the liquid, resulting in greater changes in signal intensity. In contrast, larger particles experience more friction and move more slowly, which in turn results in similar positions at different time points and produces less variation in signal

intensity.¹¹⁵ This characteristic is correlated with the diffusion coefficient of particles and influenced by the viscosity of the medium, temperature, and size of particles.^{112, 113, 115}

The hydrodynamic radius (R_h) of particles—which is a hypothetical value calculated by assuming the particle as an ideal hard sphere, is derived from the autocorrelation function of the resulting spectrum of light diffusion intensity and undergoes the Stokes-Einstein equation (**Equation 1.1**).^{112, 115}

$$D = \frac{k_B T}{6\pi\eta R_h} \quad \text{Equation 1.1}$$

where D is the diffusion coefficient, η is the viscosity of the medium, T is the absolute temperature, and k_B is the Boltzmann constant ($1.380 \times 10^{-23} \text{ kg.m}^2.\text{s}^{-2}.\text{K}^{-1}$).

For polydisperse samples, multiple peaks may appear, each with its mean size; however, the Z-average is reported as a single value (**Figure 1.6**).¹¹²

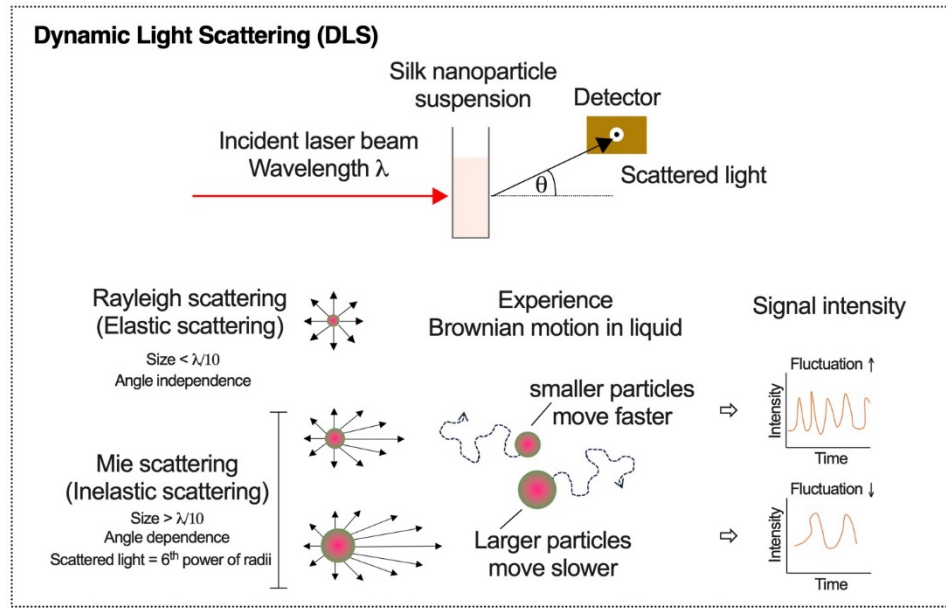


Figure 1.6 Schematic showing the principle of Dynamic Light Scattering (DLS), adapted from^{112, 116}

1.2.3.3. Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis (NTA) is a technique used to measure nanoparticle size for 10–1000 nm diameter particles.¹¹⁷ Particle size analysis of NTA is performed on individual particles,¹¹⁷ whereas DLS analyzes particles collectively as an ensemble.¹¹² NTA offers advantages over DLS due to its higher resolution for polydisperse samples and the ability to provide directly visualized data, making aggregation phenomena readily observable.¹¹⁸

NTA uses lasers to illuminate particles that move according to Brownian motion in a medium, flowing under the microscopic objective lens. The scattered light is collected by a lens.¹¹⁷ The captured video is used to analyse particle size, size distribution, and concentration.¹¹⁹ The analysis software processes the video, enabling the individual localization and tracking of each particle center. This process is to generate the mean squared distance travelled between adjacent frames ($\overline{(x,y)^2}$), which enables the calculation of the diffusion coefficient (D) (**Figure 1.7**).

$$D_t = \frac{\overline{(x,y)^2}}{4} \quad \text{Equation 1.2}$$

where t is a time period between the start of adjacent frames. The particle size—as a sphere-equivalent hydrodynamic diameter (D_h)—is calculated according to the Stokes-Einstein equation, as follows.

$$\overline{(x,y)^2} = \frac{4k_B T}{3\pi\eta D_h} \quad \text{Equation 1.3}$$

where η is the viscosity of the medium, T is the absolute temperature, and k_B is the Boltzmann constant ($1.380 \times 10^{-23} \text{ kg.m}^2.\text{s}^{-2}.\text{K}^{-1}$).¹¹⁷

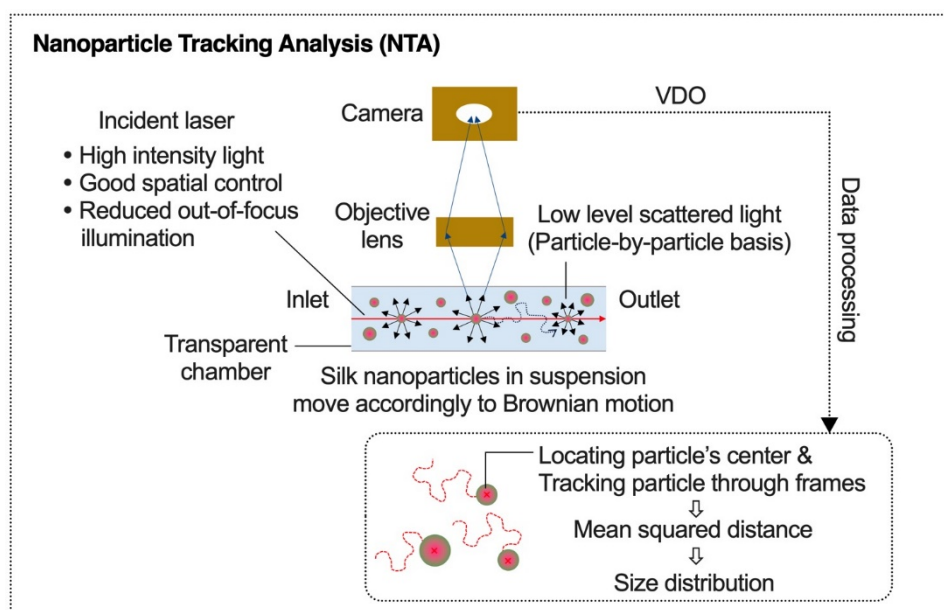


Figure 1.7 Schematic showing the principle of Nanoparticle Tracking Analysis (NTA), adapted from¹¹⁷

1.2.3.4. Electrophoretic Light Scattering (ELS)

Electrophoretic light scattering (ELS) is a routine technique for measuring the electrophoretic mobility of particles in suspension. Electrophoretic mobility is converted into zeta potential, which represents the electrical potential at the hydrodynamic slipping plane surrounding each particle.^{120, 121} The zeta potential indicates the charge on particles and their behaviour in suspension due to electrostatic interaction, establishing the basis for particle stabilization strategies in dispersion.¹²¹

ELS is used to measure the electrophoretic mobility of particles, in which particle motion influenced by the external electric field shifts the frequency of light scattering, owing to the Doppler effect—a change in frequency related to the velocity of the particles.^{120, 122} Therefore, the electrophoretic mobility is calculated as a function of the drift velocity and the magnitude of the electric field,^{120, 123} as follows.

$$v_d = m_e E \quad \text{Equation 1.4}$$

where v_d is the drift velocity ($\mu\text{m/s}^{-1}$), E is the magnitude of the electric field (V/cm^{-1}), and m_e is the electrophoretic mobility ($\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$) (**Figure 1.8**).

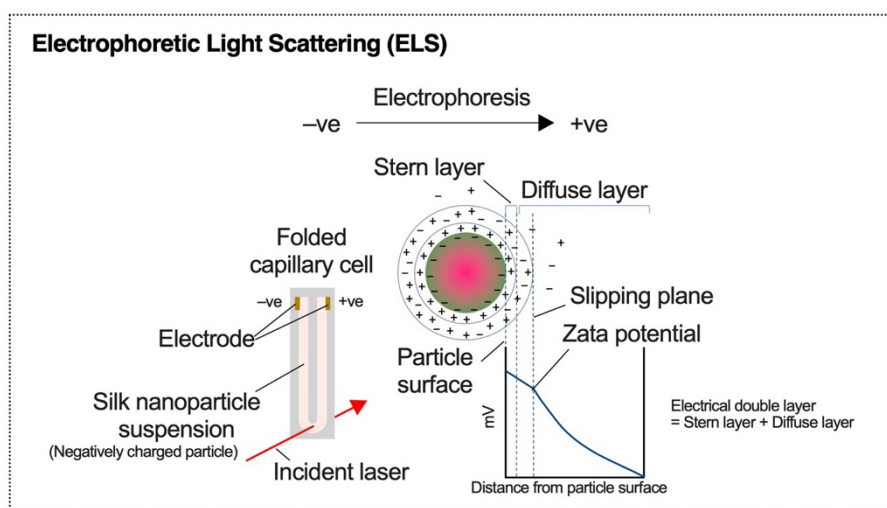


Figure 1.8 Schematic showing the principle of Electrophoretic Light Scattering (ELS), adapted from^{121, 123}

1.2.3.5. Field Emission Scanning Electron Microscopy (FE-SEM)

Field emission scanning electron microscopy (FE-SEM) is a variant of scanning electron microscopy (SEM), contributing to high-sensitivity detection and high-resolution imaging of various specimens.^{124, 125} Typically, the SEM technique relies on the detection of electrons generated during the interaction between the electron beam and the specimen surface. The high-voltage electron beam (0.3–30 kV), known as the primary electron beam, interacts with the sample surface, emitting secondary electrons (SE) at the contact area. Meanwhile, primary electrons penetrate deeper into the sample, generating higher-energy backscattered electrons (BSE). The imaging process involves the detection of emitted electrons, where the number of electrons collected for each pixel determines the image contrast (**Figure 1.9**).^{126, 127}

To date, several studies on silk nanoparticles have reported FE-SEM and/or other SEM sub-techniques as a powerful tool for silk nanoparticle analysis, specifically for size and morphology assessment,¹²⁸ aggregation behaviour,¹²⁹ as well as incorporated functions with other techniques such as DLS^{130, 131} and NTA.¹³² However, this thesis aims to further integrate its application with field-flow fractionation (FFF) (section 1.2.3.7), a technique with no

previous reports of its use in research on silk nanoparticles. Therefore, this approach may provide more detailed insights, enabling future development of the design and optimization of silk nanoparticles.

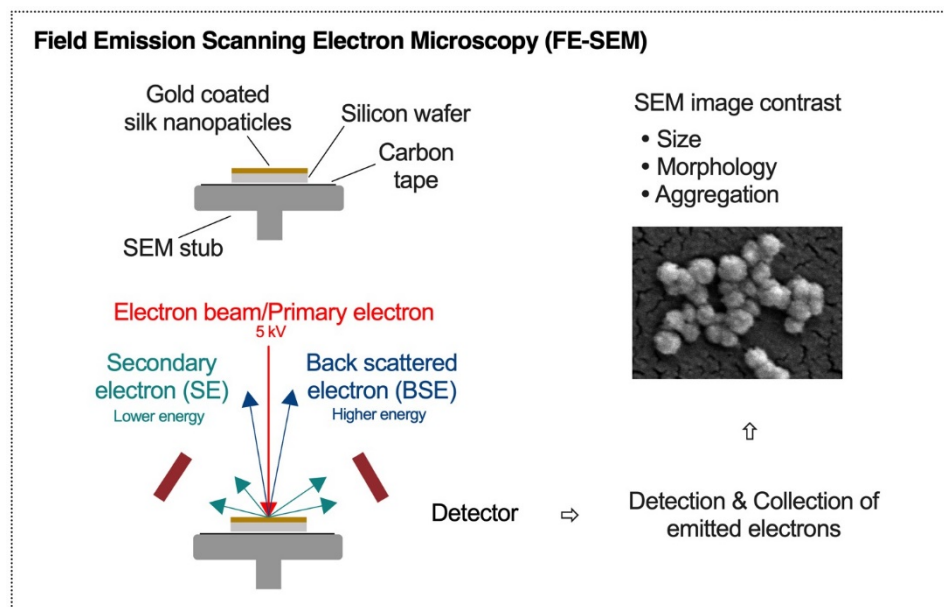


Figure 1.9 Schematic showing the principle of Field Emission Scanning Electron Microscopy (FE-SEM), adapted from^{126, 127}

1.2.3.6. Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR)

Spectroscopy

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) is a surface-sensitive technique that measures absorbance from the sample layer in direct contact with the ATR crystal, rather than from the entire bulk material; therefore, providing an opportunity to enhance the sensitivity of measurement with multiple ATR.¹³³

The general principle of FTIR spectroscopy relies on chemical bonds associated with different functional groups exhibiting unique molecular vibrations, resulting in a distinct IR spectrum.¹³⁴ In the mid-IR region ($4,000\text{--}1,000\text{ cm}^{-1}$), two primary types of molecular vibrations occur: (1) stretching vibrations (ν)—which involve changes in bond length, and (2)

bending vibrations, which involve variations in bond angles are further classified as in-plane (δ) and out-of-plane (γ).¹³⁴

FTIR has been reported as a sensitive technique for protein analysis, providing information for structural analysis by identifying the main absorption bands, including Amide I ($1600\text{--}1700\text{ cm}^{-1}$)^{133, 135} and Amide II ($1500\text{--}1600\text{ cm}^{-1}$)¹³³—which represent the peptide bond of the protein backbone.¹³³ The Amide I band corresponds mainly to the stretching vibrations of the C=O bond and weakly bending vibrations of C–N and N–H bonds,^{133, 136} while the Amide II band is primarily a consequence of the bending vibrations of the N–H and C–N bonds.^{133, 136} Because these bonds are involved in hydrogen bonding during secondary structure formation, the characteristics of the Amide I and Amide II bands are typically used as indicators to probe the secondary structure of proteins.¹³⁶ The Amide I band has emerged as the most conformation-sensitive region for proteins, showing strong responsiveness to IR light.¹³³ Differences in backbone conformation and hydrogen bonding pattern within each secondary structure (e.g., α -helix, β -sheet, β -turn, and random coil) result in distinct characteristic frequencies and intensities in the amide I region (**Figure 1.10**).^{133, 135} Recently, ATR-FTIR has been widely used in studies of silk secondary structure, serving various purposes such as investigating structure–function relationships influenced by synthesis methods²³ and pharmaceutical loading,¹³⁷ assessing pharmaceutical release potential,¹³⁸ and evaluating biodegradation behaviour.⁶

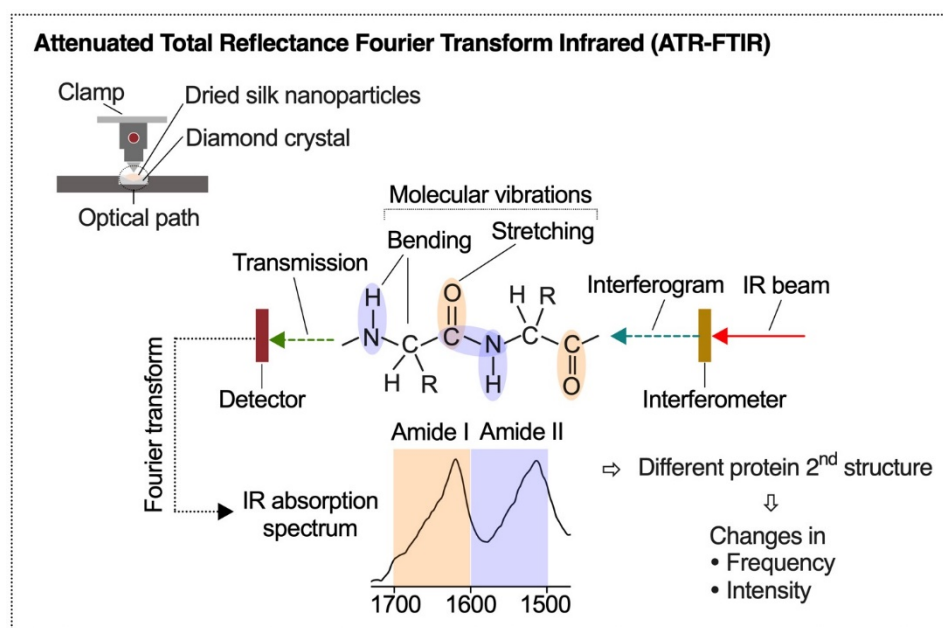


Figure 1.10 Schematic showing the principle of Attenuated Total Reflectance Transform Infrared (ATR-FTIR), adapted from^{133, 135, 136}

1.2.3.7. Flow Field-Flow Fractionation (FFF)

1.2.3.7.1 Asymmetric Flow Field-Flow Fractionation (AF4)

Asymmetric flow field-flow fractionation (AF4), an FFF sub-technique, has gained significant attention in the development of nano-sized pharmaceuticals, offering excellent intrinsic separation capability as a new technology for nanoscale characterization, isolation, and purification.¹³⁹⁻¹⁴² The most frequently used FFF operation modes are categorized as normal, steric, and hyperlayer modes, each based on distinct separation mechanisms. This thesis focuses on the normal mode operation, which is well-suited for the separation of macromolecules and submicron-sized particles ($< 1 \mu\text{m}$).^{140, 143}

Typically, the AF4 channel comprises two flat blocks, a spacer, a permeable membrane, and a porous frit, and is termed “asymmetric flow” because a single inlet stream is divided into channel and cross flows—owing to the absence of an upper permeable membrane—with their ratio governed by controlled in-line flow resistances (**Figure 1.11a**).¹⁴⁴

The separation mechanism in normal mode can be described as the following sequence (**Figure 1.11b** and **Figure 1.11c**):

(1) Focusing: Particles are first introduced into a chamber called the “channel,” where a narrow sample band is formed by a secondary input flow known as the “focusing flow.” This focusing flow enters near the centre of the channel and splits into two streams flowing in opposite directions: one opposes the injection flow to retain the sample band at a defined position, while the other flows toward the outlet to maintain a constant detection flow during this step. The injection flow and focusing flow, which converge to stack the sample, exit the channel as a “cross flow” directed downward toward the permeable membrane.¹⁴⁴⁻¹⁴⁶

(2) Relaxation: Once a particle enters the channel, the externally generated “cross flow” drives the particles to move forward and get closer to the lower permeable membrane, also called the accumulation wall, with different speeds accorded to size, yielding a concentration gradient of particles in which the smaller sizes exhibited a greater diffusivity away from the membrane.^{139, 143, 145, 146}

(3) Elution: The elution occurs after the focusing and relaxation period, during which the externally generated “cross flow” remains applied. Once the balance between those two opposite transports, smaller particles with greater elevation will diffuse more rapidly into the parabolic flow profile, with a mobile phase flow occurring inside the channel toward the output, and requiring a shorter retention time (RT) to exit the channel and undergo detection; therefore, the smaller particle size corresponds to a shorter RT.^{139, 143, 145, 146}

(4) Detection: Multiple inline detectors have proven to be compatible with AF4. This thesis employs several detectors, including a Multi-Angle Light Scattering (MALS) detector, an Ultra-Violet (UV) detector, a fluorescence detector, and an inline Dynamic Light Scattering (DLS) (**Figure 1.6**).

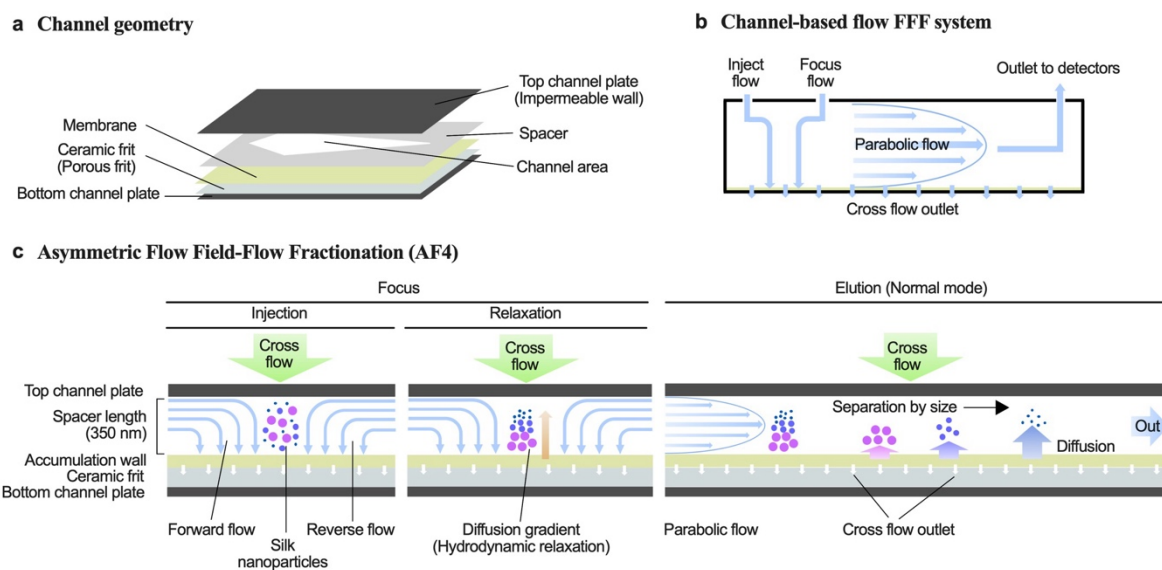


Figure 1.11 Illustration of (a) channel geometry, (b) flow system for Flow Field-Flow Fractionation (FFF), and (c) Schematic of silk nanoparticle analysis approaches using the Asymmetric Flow Field-Flow Fractionation (AF4), adapted from¹⁴⁴

1.2.3.7.2 Electrical Asymmetric Flow Field-Flow Fractionation (EAF4)

Electrical Asymmetric Flow Field-Flow Fractionation (EAF4) combines AF4 with electrical FFF (ElFFF) into one channel with a two-force FFF,¹⁴¹ facilitating the measurement of electrophoretic mobility (μ) of individual populations as a function of particle size,^{147, 148} together with improving particle size selectivity.¹⁴¹ EAF4 requires electrodes to generate an externally generated field, an electric field, influencing the separation of charged particles due to their distinct electrophoretic mobilities and diffusion coefficients.¹⁴¹ Due to the high compatibility of EAF4 with high ionic strength mobile phases, EAF4 has emerged as a technique for analysing proteins under physiological conditions.¹⁴⁸

The separation mechanism of EAF4 is similar to AF4; however, its key distinguishing features are reliance on an externally generated field called “electric field” as the external force.¹⁴¹ The positive top electrode accelerates the movement of particles towards the membrane based on their size and charge density, while simultaneously increasing the negative surface charge of the membrane. In the case of negatively charged particles, this

results in a shorter RT due to increased electrostatic repulsion between the analyte particles and the FFF membrane.^{147, 149} Conversely, applying a negative current to the top electrode alters the particle-membrane interaction, increasing the electrostatic attractive force generated by the bottom electrode with negatively charged particles, which in turn leads to a longer RT.¹⁴⁹ The behaviour of positively charged particles can be explained as following the opposite trend of that observed for negatively charged particles, as described above, due to the charge properties (**Figure 1.12**).¹⁴⁷

Electrical Asymmetric Flow Field-Flow Fractionation (EAF4)

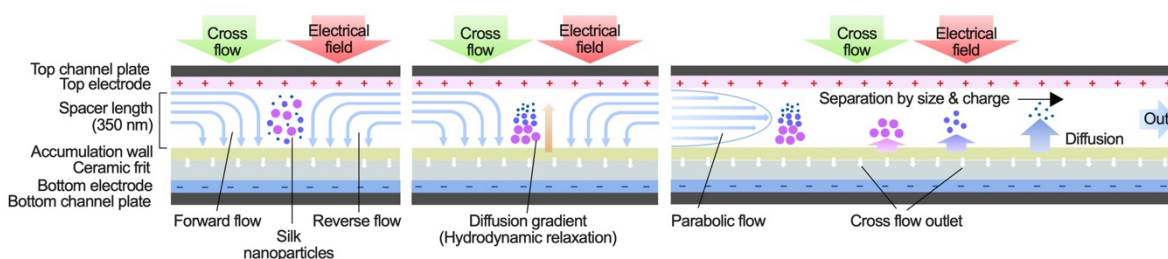


Figure 1.12 Schematic of silk nanoparticle analysis approaches using the Electrical Asymmetric Flow Field-Flow Fractionation (EAF4).

1.2.3.7.3 Frit-Inlet Asymmetric Flow Field-Flow Fractionation (FI-AF4)

Frit-Inlet Asymmetric Flow Field-Flow Fractionation (FI-AF4) is a variant of AF4. Typically, focusing is a crucial step in AF4, offering complete hydrodynamic relaxation of the particles. Therefore, the lack of a focus flow in FI-AF4 allows the high-speed frit flow to facilitate hydrodynamic relaxation of the particle-containing streams during the elution process. Therefore, FI-AF4 overcomes limitations of conventional AF4, such as preventing particle aggregation, reducing particle loss due to membrane adsorption, eliminating complete sample relaxation by skipping the focusing step, minimizing sample overloading, and enabling high sample mass injection (**Figure 1.13**).^{146, 150}

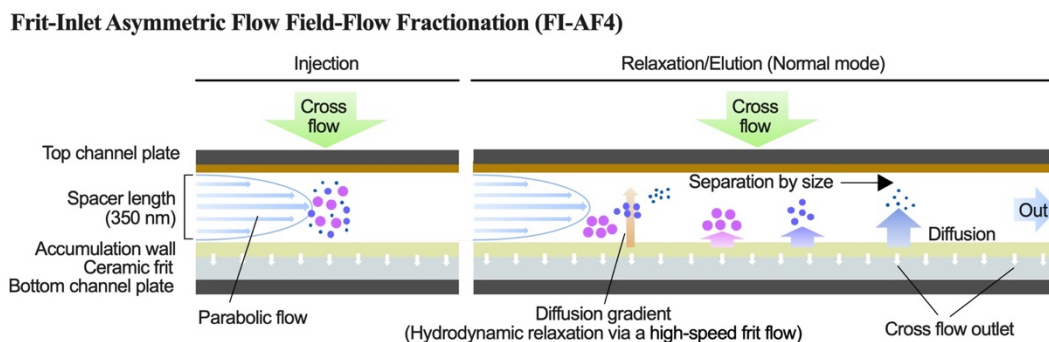


Figure 1.13 Schematic of silk nanoparticle analysis approaches using the Frit-Inlet Asymmetric Flow Field-Flow Fractionation (FI-AF4).

1.3. Challenges in Developing Silk Nanoparticles for Targeted Cancer Treatment

Tumours are neoplasms characterized by aberrant cellular proliferation arising from genetically related cells. This uncontrolled growth is frequently accompanied by metabolic dysregulation and host immune responses.^{151, 152} The term “cancer” specifically denotes tumours with the capacity to invade adjacent normal tissues, distinguishing malignant tumours from their benign counterparts based on this invasive potential.¹⁵³

1.3.1. Potential Anticancer Agent: Doxorubicin

Doxorubicin is a widely recognised with broad-spectrum efficacy across various cancer types. As a topoisomerase II inhibitor, doxorubicin targets an enzyme that is abundantly expressed in many malignancies.¹⁵⁴ Classified as a non-selective class I anthracycline,^{154, 155} its chemical structure is composed of an anthraquinone ring that intercalates between two base pairs and double-strand DNA and stabilized with DNA bases by hydrogen bonding, linked with the daunosamine domain *via* a glycosidic bond that enables groove binding.¹⁵⁵

The PEGylated liposomal doxorubicin (PLD), a model nanomedicine for doxorubicin delivery, was approved by the Food and Drug Administration in 1995, due to its high efficiency as a monotherapy with a low dose (10–20 mg/m²) for Kaposi sarcoma lesions, with evidence that increased permeability and retention (EPR) effect. Readout from a phase III study

demonstrated a 30% reduction in death, culminating in PLD approval for the indication in ovarian cancer in 2001.¹⁵⁶

PEGylated liposome DOX (Doxil®) (Janssen Pharmaceuticals, Inc., Beerse, Belgium), a specifically developed PLD, has also become a ray of hope as a nanomedicine product, offering patients a reduced doxorubicin-induced cardiotoxicity, a five-fold increase in drug and tumor exposure half-life, and prolonged clearance compared to the free drug.^{2, 157, 158} However, major concerns remain over the adverse effects caused by PLD-based nanomedicine products, as well as conventional and non-PLD formulations.¹⁵⁹ Therefore, alternative delivery strategies continue to be in demand to overcome these limitations in a strive to improve patient outcomes.

1.3.2. Silk Nanoparticles as a Promising Platform for Anticancer Drug Delivery

Nanomedicines circulate in the bloodstream to enable tumour accumulation while minimising toxicity to healthy tissue. Their physicochemical attributes including size, surface charge, and morphology, mechanical deformability have gained significant attention and must be carefully considered, as they influence tumour accumulation.¹⁶⁰ The infiltration of nanoparticles into tumour tissue occurs through endothelial gaps and/or by crossing the endothelial barrier with the assistance of neutrophils. This process is also influenced by the tumour microenvironment (TME), which presents high interstitial pressure within tumour tissues and the extracellular matrix (ECM) where drug-ECM interactions may dominate and hinder nanomedicine diffusion. Together, these factors obstruct the diffusion of nanoparticles from blood vessels into tumor tissue.¹⁶⁰

Nanoparticles in the 10–200 nm size range exhibit extended circulation time and reduced uptake by the reticuloendothelial system (RES),¹⁶¹ thereby enhancing passive accumulation in tumour tissues *via* the enhanced EPR effect;^{161, 162} This effect is caused by the presence of an endothelial gap, leading to nanoparticle leakage and accumulation in the TME,

together with a lack of lymphatic drainage that prolongs the retention of the nanoparticles.¹⁶³ Furthermore, morphology (e.g., spherical, rod-shaped, or discoidal) plays an important role in cellular uptake, offering different contact functions with biological components. For example, spherical particles facilitate optimal circulation and biodistribution. In addition, nanoparticle surface properties influence interactions between nanoparticles and biomacromolecules in the circulatory system.^{161, 164}

Silk nanoparticles have emerged as a challenging strategy for cancer treatment, with the ultimate purpose of (1) improving intratumoral accumulation through altered biodistribution: carrying the drug to the target site specifically and effectively while minimizing drug exposure to healthy tissue—which reduces toxicity and adverse events in the patient;¹⁶⁵ (2) improving drug efficacy: increasing drug solubility, bioavailability, loading, stability, and circulation time, promoting sustained drug release and improving patient adherence by reducing the frequency of day hospital admissions for drug administration;^{165, 166} (3) enabling targeted therapy: to increase cancer cell selectivity;^{167, 168} (4) allowing for combination therapy for multiple payloads: encapsulation of multiple agents—which may evade chemoresistance and enhance efficacy;^{169, 170} and (5) contributing to the development of personalized medicine: designing specific cancer therapy to match cancer types and individual genetic profiles.^{170, 171} **Table 1.2** summarises silk nanoparticle formulations under pre-clinical investigation, providing details on manufacturing methods with various payloads, physicochemical properties—specifically, size, morphology, and charge, and their intended applications in cancer therapy.

Table 1.2 Anticancer agent-loaded silk nanoparticles (*Bombyx mori* silk) fabricated by antisolvent precipitation and their desired function in cancer therapy.

Manufacture method/Detail	Anticancer agent encapsulation			Physicochemical attributes			Desired function	Reference
	Anticancer agent	Drug loading (%)	Encapsulation efficiency (%)	Diameter (nm)	ZP (mV)	Shape		
Antisolvent precipitation with incorporation of crosslinking agents	α -Mangostin	4–7	40–80	~300	–15 to +30	Spherical	Enhancing cytotoxicity towards Caco-2 and MCF-7 cell lines	¹³⁷
Antisolvent precipitation	Docetaxel trihydrate	37–47	56–72	178–198	–27 to –19	Spherical	Improving drug solubility and enhancing cytotoxicity against MCF-7 and MDA-MB-231 breast cancer cell lines	¹⁷²
Antisolvent precipitation Adsorption with surface modifier (Tween-80) and drug	Doxorubicin hydrochloride	–	85	131	–17	Spherical	Improving retention and penetration through the blood brain barrier	¹⁶⁴
Antisolvent precipitation with zeolitic imidazolate framework-8 (ZIF-8)/Adsorption with drug	Doxorubicin hydrochloride	5	86	~100	+17	Spherical	Improving novel pH-responsive drug delivery system	¹⁷³
Antisolvent precipitation	Tamoxifen citrate	–	79	186	–20	Spherical	Enhancing cytotoxicity against MCF-7 and MDA-MB-231 breast cancer cell lines	¹³⁰
Antisolvent precipitation with doxorubicin, surface modification with polyethylenimine, adsorption with survivin siRNA	Doxorubicin hydrochloride and survivin siRNA	6–20	65–91	204–235	–11 to –1	Spherical	Improving therapeutic efficiency using codelivery of doxorubicin and survivin siRNA in the 4T1 mouse mammary cell line	¹⁷⁴
Microfluidic device with incorporation of amphiphilic anticancer peptide (G(IKK)3I-NH ₂ or G3)	ASC-J9	–	61–84	129–232	–36 to –19	Spherical/Irregular shape	Improving selectivity on HCT 116 cancer cells for targeted delivery of the hydrophobic anticancer agent ASC-J9	¹⁷⁵

Drug loading is a crucial parameter in drug delivery systems, as low drug loading represents a major challenge for the clinical translation of nanomedicines, making dose scaling in humans impractical and economically unfeasible.¹⁷⁶ Nanocarriers with low drug loading require higher doses, which can increase off-target toxicity. In contrast, high drug loading significantly improves therapeutic efficacy by reducing the amount of nanocarrier required to deliver effective drug concentrations to the tumour site, thereby lowering excipient-related toxicity and manufacturing costs and ultimately enabling greater clinical efficacy.¹⁷⁷

Silk nanoparticles are a promising platform for cancer therapy, as they not only enable high drug loading but also facilitate sustained and stimuli-responsive drug release, which is important for enhancing therapeutic efficacy and reducing toxicity.¹⁷⁸ Unlike the physiological pH of 7.4 in blood,¹⁷⁹ the tumour microenvironment is acidic (extracellular pH 6.5–6.8). After internalization, nanoparticles encounter even lower pH in late endosomes (~6.5) and lysosomes (~4.5)^{180, 181} Therefore, silk nanoparticles with pH-triggered drug release targeting tumour sites are well-suited to meet the criteria for clinically relevant drug delivery.

Silk nanoparticles enhance therapeutic outcome in breast cancer, suggesting clinical relevance by attempting to overcome the limitations of free docetaxel (DXL), such as low water solubility and systemic toxicity.¹⁷² Silk nanoparticles achieve high drug loading and encapsulation efficiencies for 5-fluorouracil (5-FU) and demonstrate sustained release of 90% of the encapsulated 5-FU released after 110 h.¹⁸² Moreover, silk nanoparticles prepared using a microfluidics-assisted desolvation method enable efficient drug loading (82% for 5-FU and 37% for curcumin). The clinical relevance of this high-loading formulation was further evaluated through *in vitro* cytotoxicity studies against breast cancer cells and *in vivo* biodistribution analysis in a murine breast cancer model, showing significant enhanced tumour necrosis.¹⁸³ However, to date, translating silk biomaterials into effective drug delivery systems

for anticancer agents remains challenging, as issues related to scale-up manufacturing and long-term safety assessment of silk nanoparticles are still under exploration.¹⁸⁴

The exceptional properties of silk nanoparticles motivate this thesis, inspiring the rational design of ion-mediated silk nanoparticles (Ca^{2+} and K^+), as understanding the role of ions in modulating silk's structural and conformational properties could strengthen scalable manufacturing. This strategy addresses a major challenge in drug delivery, since ionic incorporation can economically enhance product yield while enabling integration into large-scale production. Therefore, investigating ion-mediated silk assembly is expected to provide mechanistic insights and guide the development of silk nanoparticles with enhanced drug loading, controlled release, and clinical translational potential.

1.4. Hypothesis, Aims, and Objectives

This thesis reviews the natural modulation of metal ions, focusing on Ca^{2+} and K^+ , in the silk gland of the silkworm and extends this concept to *ex vivo* regulation to modulate silk nanoparticle production. This thesis hypothesizes that this biomimetic strategy used by the silkworm *in vivo* could be engineered to fine-tune the critical quality attributes of silk nanoparticles, enabling the achievement of a predetermined particle size range and production yield using the antisolvent precipitation method in a semi-batch format. The overall aim of this thesis is to (1) generate liquid silk from *B. mori* silk cocoons through chemical treatment processes, including degumming with Na_2CO_3 and dissolution with LiBr , (2) manufacture silk nanoparticles using regenerated liquid silk incorporating Ca^{2+} and K^+ *via* an antisolvent precipitation method, (3) profile their physicochemical properties and stability in various media and (4) finally explore their function as delivery systems for cytotoxic payloads.

Chapter 1 provides background knowledge of principles underpinning silk fibroin protein structure, identify gaps in current silk nanoparticles development, and highlights its promise as a biomaterial for designing advanced drug delivery systems.

Chapter 2 explores the role of Ca^{2+} in promoting silk fibroin self-assembly into nanoparticles *via* antisolvent precipitation manufacturing. The resulting physicochemical properties, such as size, surface charge, protein secondary structure, and cellular responses, are addressed. This chapter was published in ACS Biomaterials Science & Engineering, 11(3), 1847-1856. (DOI: <https://doi.org/10.1021/acsbiomaterials>).

Chapter 3 further investigates the impact of K^+ on silk nanoparticle properties in comparison to Ca^{2+} , highlighting the key attributes of the resulting nanoparticles as a promising drug nanocarrier candidate. This chapter was published in ACS Applied Bio Materials, 8(8), 6854-6864. (DOI: <https://doi.org/10.1021/acsabm.5c00598>).

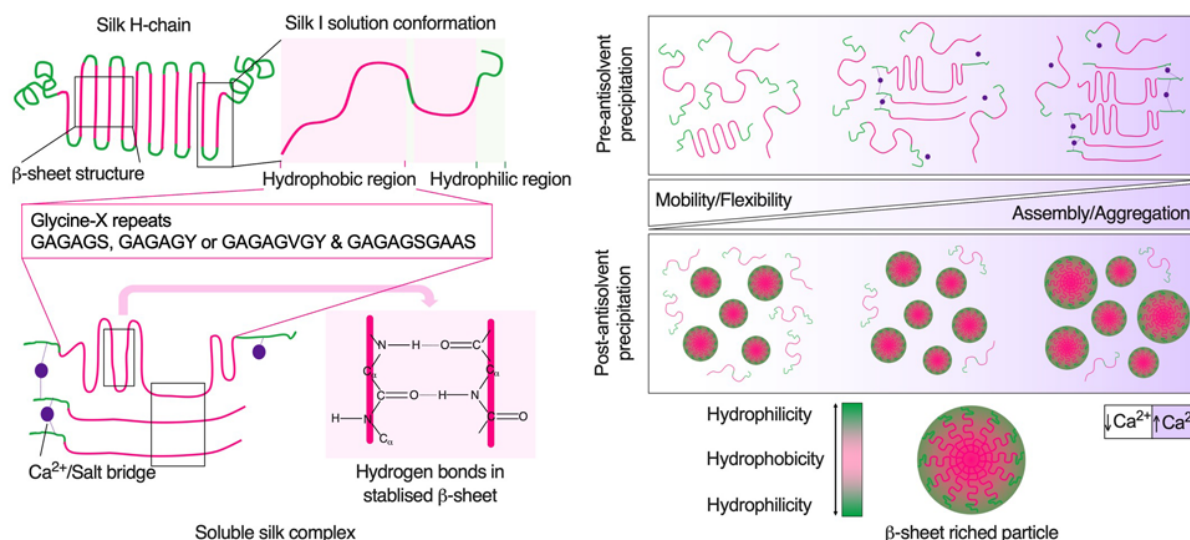
The physicochemical stability of silk nanoparticles generated with different cationic additives during nanoprecipitation remains an unexplored area. **Chapter 4** addresses this gap through the evaluation of the short- and long-term physicochemical stability of Ca^{2+} and K^+ silk nanoparticles in solution, together with the use of the EAF4-UV-MALS-DLS and orthogonal analytical techniques to measure changes in their physical attributes over a 56-day period. This chapter was published in Nanoscale Advances, 7(18), 5519-5535. (DOI: <https://doi.org/10.1039/D5NA00365B>).

Chapter 5 investigates the doxorubicin loading and release behaviour of doxorubicin- loaded silk nanoparticles, as influenced by the antisolvent precipitation and adsorption methods for drug loading. Their comparative physical attributes were quantified with orthogonal techniques, along with their drug loading content and *in vitro* release kinetics. This chapter excludes the investigation of Ca^{2+} - and K^+ -mediated silk nanoparticles, as its primary objective was to establish a foundational assessment of silk nanoparticles as carriers

for doxorubicin before extending the investigation to ion systems. **Chapter 6** provides a general discussion of outcomes of this thesis and the future directions arising from this research.

For the proposed intravenous route of administration, the principal critical quality attributes (CQAs) investigated in this thesis are particle size, surface charge, physicochemical stability, drug loading, and drug release behaviour. These parameters were selected because of their direct influence on formulation quality and performance, in line with the aim of this thesis to develop and characterize silk nanoparticle formulations. Further evaluation of formulation safety, sterility assurance, endotoxin contamination, injectability, systemic compatibility, and therapeutic performance following intravenous administration will be required in the future development of silk nanoparticles.

Chapter 2 Biomimetic silk nanoparticle manufacture: calcium ion-mediated assembly



Chapter 2 details the results from the published article in ACS Biomaterials Science & Engineering (Roamcharern, N.; Matthew, S. A. L.; Brady, D. J.; Parkinson, J. A.; Rattray, Z.; Seib, F. P. Biomimetic silk nanoparticle manufacture: calcium ion-mediated assembly, ACS biomaterials science & engineering 2025, 11(3), 1847-1856. <https://doi.org/10.1021/acsbiomaterials.4c02175>). In this work, I utilized the silk nanoparticle manufacture optimized by Dr. Saphia Matthew, selecting her best condition of antisolvent precipitation using a semi-batch format: dropwise to isopropanol at a syringe-pump speed of 1 mL/min, a height of 7.5 cm, and a stirring speed of 400 rpm. I optimized the Ca²⁺ mass ratio used in nanoparticle synthesis, designed and performed experiments used in nanoparticle analyses (e.g., ThT assay, FTIR, DLS, ELS, NTA, FE-SEM, yield determination, and cytotoxicity and inflammation assay). In collaboration with Dr. John Parkinson and Dr. Daniel Brady, I conducted the NMR study using modified isotope-labeled silk cocoons prepared by Dr. Brady. The experimental design of NMR experiments and data interpretation were carried out with the assistance of Dr. Parkinson. The entire chapter was supervised by Prof. Zahra Rattray and Prof. Philipp Seib.*

2.1. Introduction

Silk has emerged as a promising biopolymer for expanding the repertoire of bioinspired drug-delivery carriers that presently include collagen,¹⁸⁵ chitosan,¹⁸⁶ and cellulose,¹⁸⁷ among others. Silk serves as a useful benchmark for biomedical applications by virtue of its favorable biodegradability, biocompatibility and protecting payload from degradation.¹⁸⁸⁻¹⁹⁰ Silk biomolecular characteristics are fundamentally governed by its primary amino acid sequence and orchestrated assembly, which result in an overall hierarchal structure. Controlling this hierarchal structure broadens the range of silk applications to tissue regeneration and as silk-based drug delivery applications.²

At the molecular level, *Bombyx mori* silk is composed of a high-molecular-weight heavy chain (H-chain, 390 kDa), a low-molecular-weight light chain (L-chain, 26 kDa), and the glycoprotein fibrohexamerin (formally P25) (30 kDa). The H-chain possesses amorphous regions (β -turns, α -helixes, and random coils) and a crystalline region (β -sheet), which account for 33% and 66% of the total sequence, respectively.¹¹ Capping the silk sequence at the N- and C-termini of the H-chain are non-repeating amino acid residues. The hydrophobic block of the H-chain is a repetitive sequence of glycine-X (GX) repeats, where X is alanine (A), serine (S), or tyrosine (Y) and accounts for 65%, 23%, and 9% of the sequence, respectively. These GX repeats are ordered by three key sequences: i) GAGAGS, ii) GAGAGY or GAGAGVGY, and iii) GAGAGSGAAS. The H-chain is composed of 12 hydrophobic blocks linked together by 11 hydrophilic blocks of amorphous regions, each consisting of 31 amino acids. Each repetitive sequence of the H-chain hydrophobic blocks governs different structural characteristics. The GAGAGS sequence contributes to the bulk of the crystalline regions and is typically located at the beginning of each motif, whereas the GAGAGY or GAGAGVGY sequence forms semi-crystalline regions, and GAGAGSGAAS acts as a sheet-breaking motif.^{2, 24, 191, 192}

In the silk gland, the structure of the silk protein is in part modulated by ions, which guide structural transformation from the liquid storage form (amorphous silk I) to the “spinning ready” form. Calcium ion (Ca^{2+}) levels decrease from the posterior to the anterior segments of the middle silk gland, from approximately 3,000 to 500 mg/g dry luminal content weight.²⁸ In the presence of Ca^{2+} , a salt bridge is formed between Ca^{2+} and hydrophilic groups of the silk molecule. These salt bridges stabilize the silk conformation during storage, thereby increasing the silk solution viscosity to present a gel-like liquid silk state.^{17, 28}

Investigations into the conformational and interactional dynamic alterations of silk are now increasing our knowledge of the control of silk structure before and after silk fibre spinning.^{193, 194} Various analytical techniques, such as nuclear magnetic resonance (NMR) and Fourier-transform infrared (FTIR), are routinely deployed for the characterization of silk.^{13, 17, 26, 193-195} Solid and solution-state NMR has been used to classify silk structure and the conformational transitions that dictate the physicochemical properties of each unique repeated sequence.^{11, 193} This information can be harnessed to engineer silks with favourable characteristics. For example, FTIR-based studies on the secondary structure and conformational changes occurring in Ca^{2+} -mixed *Antheraea pernyi* silk fibroin showed differences in the absorption bands (amide I-VI; 1655 cm^{-1} , 1545 cm^{-1} , 892 cm^{-1} , and 1270 cm^{-1}) over time associated with the β -sheet degree.¹⁸ These changes have important consequences in the fabrication of silk drug delivery systems, as they affect the solubility and, therefore, the likelihood of precipitation of silk to form silk nanoparticles.

Antisolvent nanoprecipitation is one of the least energy-intensive laboratory-scale methods, as it requires no complex procedures or expensive apparatus, and relies simply on the spontaneous self-assembly of proteins to form nanoparticles (25–180 nm).¹⁹⁶ Therefore an increasing number of studies are adopting this approach to manufacture silk nanoparticles.^{190, 197} For example, a semi-batch procedure involving the drop-by-drop addition of reverse-

engineered silk solution into isopropanol induced structural changes in which the low solubility of the hydrophilic blocks of the H-chain resulted in the self-assembly of β -sheet-rich silk nanoparticles (~ 100 nm).¹⁹⁶ Subsequent method refinements^{90, 131} and the use of liquid silk stocks with different molecular weight characteristics¹³¹ demonstrated the ability to tune nanoparticle properties. Despite this, the implementation of biomimetic principles to orchestrate silk nanoparticle self-assembly and improve yields have not been studied to date. The aim of the present study was to evaluate the impact of physiologically-relevant calcium ion concentrations on the critical quality attributes of silk nanoparticles manufactured by alcohol-mediated nanoprecipitation in a semi-batch format while titrating the calcium ion concentration. I hypothesised that this biomimetic approach could fine-tune the critical quality attributes of silk nanoparticles to achieve a pre-determined particle size range and a greater production yield of silk-based nanocarriers.

2.2. Materials and Methods

2.2.1. Materials

All reagents and solvents were purchased from Sigma-Aldrich or Acros Organics ($>98\%$ purity) and used without additional purification unless noted. *B. mori* silk cocoons were purchased from Scientific Center on Sericulture, Vratsa, Bulgaria.

2.2.2. Aqueous Silk Preparation and Silk Nanoparticle Synthesis

The procedures used to manufacture silk nanoparticles have been described elsewhere.²³

Figure 2.1 shows the workflow used for silk nanoparticle and aqueous silk fibroin preparation (referred to as silk in the manuscript). Briefly, *B. mori* silk cocoons were chopped into 5×5 mm pieces. Next, 5 g of cocoon pieces were degummed by boiling in 2 L of 0.02 M Na_2CO_3 for 1 h under gentle stirring. Degummed silk was rinsed with 200 mL of deionised water before

immersion in 1 L of DI water for 20 min. The washing step was performed twice more, squeezing the fibers to remove excess liquid, and then drying them overnight at ambient temperature in a fume hood.

The dry degummed silk was dissolved in a warm solution of 9.3 M LiBr with a silk:LiBr ratio of 1 g to 4 mL, and then incubated at 60 °C for 4 h. The resulting SF was dialyzed against deionised water (Slide-A-Lyzer 3.5K Dialysis Cassette G2, Thermo Scientific, Rockford, IL, USA) for 48 h, centrifuged three times at 2885g for 40 min at 4 °C (PK 121R Centrifuge, rotor T515, ACL International Srl, Milan, Italy), and stored at 4 °C until further analysis. A 500 µL aliquot of SF was dried at 60 °C for 48 h for % *w/v* calculations.

Silk nanoparticles were manufactured using a drop-by-drop approach in a semi-batch format. First, silk was mixed with CaCl₂ solution to yield 6 mL of 3% *w/v* silk containing a final mass ratio of Ca²⁺:silk at 0.7 and 11.5 mg/g. The mixture was then added dropwise to isopropanol at a syringe-pump speed of 1 mL/min, a height of 7.5 cm, and a stirring speed of 400 rpm. The resulting silk nanoparticles were centrifuged at 48,400g at 4 °C for 2 h, followed by sonication in deionised water. The centrifugation and sonication cycles were repeated twice more to ensure the complete removal of contaminants. Silk nanoparticles were produced in triplicate using different silk batches (*n* = 3).

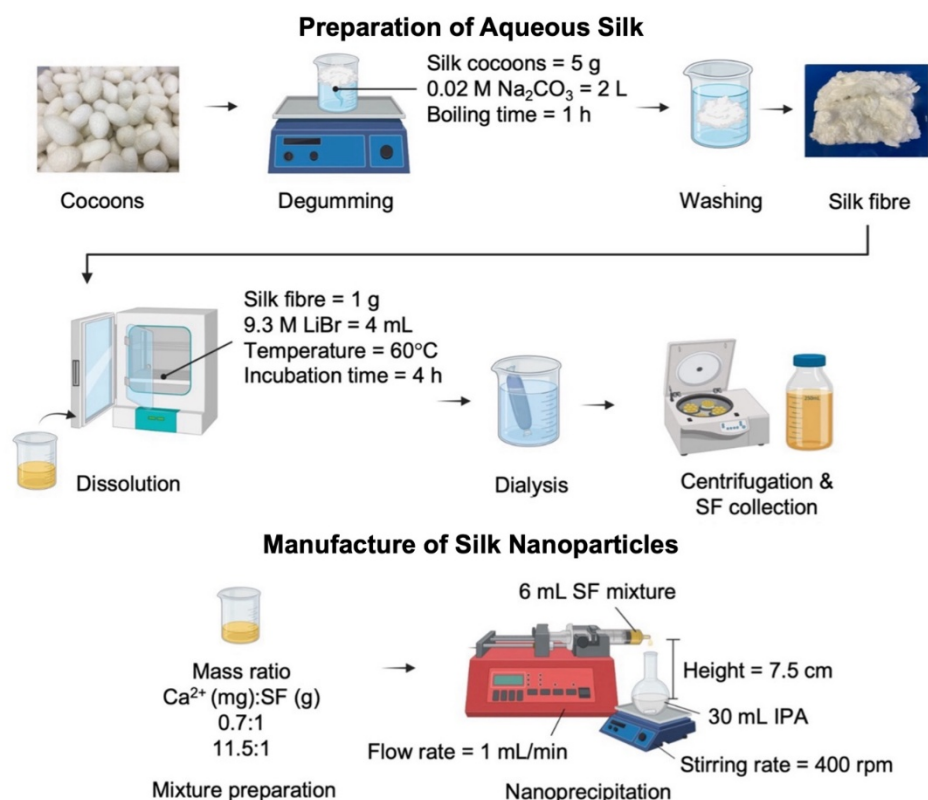


Figure 2.1 Schematic of aqueous silk fibroin preparation and silk nanoparticle manufacture.

2.2.3. Fourier-Transform Infrared Spectroscopy

The secondary structure of silk and the silk nanoparticles was determined by Fourier-transform infrared (FTIR) spectroscopy. All samples were prefrozen at $-20\text{ }^{\circ}\text{C}$ overnight prior to lyophilization at $-10\text{ }^{\circ}\text{C}$ and 0.140 mbar for 24 h. Freeze-dried silk and 70% ethanol-treated silk films were prepared as a reference for silk I and silk II crystalline structures, respectively. FTIR measurements were collected over the wavenumber range of $400\text{--}4000\text{ cm}^{-1}$ with a 4 cm^{-1} resolution (ATR-equipped TENSOR II FTIR spectrometer, Bruker Optik GmbH, Ettlingen, Germany). The scanning time was set at 32 and 128 scans for the background and sample channel scans, respectively. The secondary structure content was deconvoluted and calculated from the FTIR absorbance spectra in the region of amide I. The following wavenumbers were referenced for the identification of each secondary structure component as

described previously with minor modification;²³ 1697–1710 cm⁻¹ for antiparallel amyloid β -sheet structure, 1660–1690 cm⁻¹ for β -turn structure,²¹ 1638–1655 cm⁻¹ for random coil structure, 1620–1635 cm⁻¹ for native β -sheet structure and 1605–1625 cm⁻¹ for intermolecular β -sheet structure. Second-derivative amide I spectra (1600–1700 cm⁻¹) were used in correlation coefficient (*R*) calculations versus the air-dried silk film, as described previously.

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2.2.4. Nuclear Magnetic Resonance Spectroscopy

For selected studies *B. mori* larvae were reared on artificial diet (CREA, Sericulture Laboratory of Padua, Italy) at 25 °C $\pm$ 1 °C and 75% relative humidity. Modified isotope-labelled silk cocoons were produced by incorporating 50 mg of D-glucose-¹³C₆ (Cortec, Les Ulis, France) per 250 mg of dry artificial diet, fed twice daily over days 4–6 of the fifth instar and allowed to spin their cocoons.

Silk material was prepared as described above using unmodified silk cocoons and modified isotope-labelled silk cocoons. The 500 mL Ca²⁺-mixed silk solution was prepared by mixing unmodified and modified silk at a 1:1.5 protein mass ratio, yielding a final 3% w/v protein concentration supplemented with 0.7 and 11.5 mg Ca²⁺ per 1 g silk.

The impact of Ca²⁺ on the NMR spectroscopy response of silk samples was determined using a Bruker 600 MHz AVANCE II⁺ NMR spectrometer operating at 600.13 MHz for proton data observation and equipped with BBO-z or TBI-z probe heads, depending on the application. All samples were equilibrated at a probe head temperature of 298 K. 1D ¹H NMR spectra were typically acquired over a frequency width of 12 kHz (20 ppm) as 16 K data points (acquisition time = 681.5 ms) for each of 64 transients and centred at a transmitter offset frequency equivalent to $\delta^1\text{H} = 4.698$ ppm. Data were acquired using a Watergate pulse sequence combined with soft presaturation to eliminate the solvent resonance. 2D [¹H, ¹⁵N]

Heteronuclear Single Quantum Coherence (HSQC) NMR data were acquired over ω_2 and ω_1 frequency widths of 6 kHz (10 ppm) and 2.4 kHz (40 ppm) and centred at frequency offsets equivalent to $\delta^1\text{H} = 4.698$ ppm and $\delta^{15}\text{N} = 120$ ppm for direct ^1H and indirect ^{15}N resonance detection, respectively. The Bruker pulse program hsqcetf3gpsi was used to acquire NMR data as 2048 data points (acquisition time = 170 ms) with 16 transients for each of 128 t_1 increments using a traditional echo-antiecho data acquisition scheme. All data were processed using TopSpin version 4.0.5 (Bruker Biospin).

2.2.5. Thioflavin T Fluorescence Measurement of Aqueous Silk Fibroin and Silk Nanoparticle

Thioflavin T was used to investigate the structural changes of aqueous silk fibroin in the presence of Ca^{2+} . Briefly, 25 μL of 3% w/v silk containing Ca^{2+} (0.7 and 11.5 mg/ 1 g silk) was incubated with an equal volume of 100 μM thioflavin T in deionised water and 50 μL of deionised water. After a 10 min incubation at ambient temperature, the bound thioflavin T fluorescence intensity was read (excitation = 440 nm, emission = 475 nm) (Polarstar Omega, BMG Labtech, Ortenberg, Germany). The 60% v/v isopropanol served as a β -sheet rich (silk II) positive control.

For model drug loading studies thioflavin T served as the payload. The thioflavin T-loaded silk nanoparticles were manufactured as described above by supplementing the liquid silk mixture with 100 μL of 1000 μM thioflavin T. The thioflavin T-loaded silk nanoparticles were collected and characterised using the nanoparticle tracking analysis (NTA 3.4 Build 3.4.003, Malvern Panalytical Ltd., Worcestershire, UK). Briefly, the 1 mg/mL suspension of thioflavin T-loaded silk nanoparticles was 500 to 5000-fold diluted in 0.2 μm filtered Milli-Q water. The particle size distribution, particle concentration, and fluorescence intensity were measured with the following capture and analysis settings; sCMOS camera, Blue 488 laser, 500 nm long pass filter, syringe pump speed at 50, and viscosity at 1.0000 cP.

2.2.6. Production Yield Determination

The silk nanoparticle production yield was calculated with **Equation 2.1**.

$$\% \text{ Yield} = \frac{\text{Concentration of silk nanoparticle suspension (\% w/w)} \times \text{Mass (g)}}{\text{Concentration of aqueous silk (\% w/v)} \times \text{Volume (mL)}} \times 100 \quad \text{Equation 2.1}$$

where, mass is the total weight of the silk nanoparticle product (g), while concentration of silk solution and volume are initial concentration (3% w/v) and initial volume (6 mL) used in anti-solvent precipitation, respectively. The % w/w was calculated as detailed in **Equation 2.2**.

$$\% \text{ w/w} = \left[\frac{(W3-W1)}{(W2-W1)} \right] \times 100 \quad \text{Equation 2.2}$$

where, W1 is the weight of empty weighing boat (mg), W2 is the weight of weighing boat containing 0.3 mL silk nanoparticle suspension (mg), and W3 is the weight of weighing boat containing 0.3 mL silk nanoparticle suspension after drying at 60 °C for ≥ 72 h (mg).

2.2.7. Size and Zeta Potential Measurements

Particle size and surface net charge were determined using dynamic light scattering and electrophoretic light scattering, respectively (Zetasizer Nano-ZS Malvern Instrument, Worcestershire, UK). Size analysis was performed using a 1:25 ratio (v/v) of 1 mg/mL silk nanoparticles to deionised water with the following settings: measurement temperature, 25 °C; count rate, 203.6 kcps; measurement duration, 70 s; and measurement position, 3 mm. The zeta potential was measured using a 1:20 ratio (v/v) of 5 mg/mL silk nanoparticles to deionised water with the following settings: temperature, 25 °C; count rate, 161.5 kcps; zeta run, 12; and measurement position, 2 mm.

2.2.8. Morphology Analysis

Silk nanoparticle suspensions were sonicated twice at 30 amplitude for 30 min and prepared at 1 mg/mL in deionised water. The silk nanoparticle suspension (20 μ L) was dropped onto 5 $\times$ 5 mm silicon wafer chip (Ted Pella, Inc., CA, USA), dried at room temperature for 24 h, and gold sputter-coated from a 35 mm height for 40 s at 0.08 mb and 30 mA (Agar Scientific

Manual Sputter Coater, Agar Scientific Ltd., Essex, UK). The silk nanoparticles were imaged by field emission scanning electron microscopy at 10,000-fold, 20,000-fold, and 60,000-fold magnifications at 5 kV (Hitachi SU6600, Hitachi High-Tech Europe GmbH, Krefeld, Germany).

2.2.9. Cytotoxicity and Inflammation Measurements

RAW 264.7 murine macrophages were purchased from ATCC (product number TIB-71™, ATCC, UK). The cells were cultured in DMEM supplemented with 10% FBS, 50 U/mL penicillin, and 50 mg/mL streptomycin. Seeded cells were processed at 80–90% cell confluency by scraping, centrifuging at 380g for 4 min, and replating at 15,000 cells/cm². After 24 h, the cells were dosed with fresh complete medium containing 31.25–500 µg/mL silk nanoparticles and incubated in a 5% CO₂ incubator at 37 °C for 48 h. Viability assays were then performed by exposing the cells to 20 µL of 5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide solution for 4 h and recording absorbance at 570 nm (Multiskan Ascent V1.24, Thermo Fisher Scientific Inc., MA, USA). Cell viability was expressed as a percentage relative to untreated control cells.

Endogenous nitrite (NO₂⁻), tumour necrosis factor alpha (TNF-α) and the cytokine profile were measured as markers of cell responses to silk nanoparticle exposure. Cells treated with complete medium containing 200 ng/mL lipopolysaccharide served as an inflammatory-activated control. The tested concentration of silk nanoparticles was 500 µg/mL. Following 24 h incubation, the medium was collected and centrifuged at 21,630g for 5 min (VWR® Micro Star 30 centrifuge, VWR International, LLC., Leicestershire, UK). The supernatant was transferred to a low-binding protein microtube and stored at -80 °C for further analysis.

Endogenous NO₂⁻ levels in the supernatants were quantified using a Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit (R&D Systems, Minneapolis, MN, USA), TNF-α levels were assayed using a Mouse TNF-α Quantikine ELISA Kit (R&D Systems,

Minneapolis, MN, USA), and semi-quantitative measurements of cytokines and chemokines were performed using the Proteome Profiler™ Array (Mouse Cytokine Array Panel A, R&D Systems, Minneapolis, MN, USA). All cell-based datasets were conducted as three independent cell experiments ($n = 3$).

2.2.10. Analysis Software and Statistical Analyses

All data were collated using Microsoft Excel version 16.61.1 (Microsoft Office 365 for Mac Software, Redmond, WA, USA). FTIR data were deconvoluted using Origin 2019b (OriginLab, Northampton, MA, USA). NMR spectra were analysed and graphed using MestReNova (Mestrelab Research, Santiago de Compostela, Spain). Semi-quantitative particle sizes and circularities were obtained from the electron microscopy images. The dot intensities on cytokine membranes were analysed using Image J (the NIH's National Institute of Mental Health, Bethesda, MD, USA). All graphing and statistical analyses were conducted using GraphPad Prism 9 (GraphPad Software, Boston, MA, USA). One-way ANOVA and Dunnett's multiple comparisons were used as statistical tests. Asterisks denote statistical significance and experimental repeats (n) are specified in each figure legend.

2.3. Results

2.3.1. Conformational Changes and Secondary Structure Composition of Aqueous Silk and Silk Nanoparticles

The fluorescence Thioflavin T assay was used to measure the degree of β -sheet content and the aggregation of silk in the presence of Ca^{2+} . The fluorescence intensity of bound thioflavin T increased with ascending Ca^{2+} levels (0.7–11.5 mg) and therefore showed similar characteristics to those of an isopropanol positive control (**Figure 2.2a**).

1D ^1H NMR spectra revealed no significant differences in chemical shifts in the NH region (8.5–7.8 ppm); however, a consistent trend of increasing peak intensity and altered peak sharpness was observed with increasing Ca^{2+} mass ratio. The spectra relative to the control (0.0 mg Ca^{2+}) for 0.7 and 11.5 mg Ca^{2+} showed an integral increase of 7% and 17.5%, respectively (**Figure 2.2b**). The 2D [^1H , ^{15}N] HSQC NMR data showed ^{15}N random coil chemical shifts of amino acids that were followed by an alanine residue, including glycine (108.8 ppm), serine (115.7 ppm), isoleucine (119.9 ppm), alanine (123.8 ppm), and an unidentified amino acid (110.8 ppm)¹⁹⁸ (**Figure 2.2c**). The overall ^1H NMR (10–0 ppm) and ^{13}C NMR (190–10 ppm) chemical shift profiles of silk were also elucidated. The IR fitting of the amide I region was elucidated for the secondary structure content calculation including the percentages of β -sheets, β -turns, and random coil structures (**Figure 2.4**).

The silk I and silk II references showed a typical amide I region, which shifted from 1640 cm^{-1} to 1620 cm^{-1} . The spectra of Ca^{2+} -mediated and control silk nanoparticles were both consistent with the crystalline silk II reference, indicating an increase in β -sheet structure (**Figure 2.2d**). The mass ratio Ca^{2+} did not affect β -sheet structure formation in the Ca^{2+} -mixed silk solution phase, which exhibited a low β -sheet content in the 17–18% range. By contrast, the silk nanoparticles showed a β -sheet-enriched content of 56–61%. No significant differences were observed among the silk nanoparticle species in overall β -sheet content, but an increased proportion of intermolecular β -sheet was observed in the high mass ratio Ca^{2+} (**Figure 2.2e**). The correlation coefficient (R), which was derived from second derivative amide I spectra and refers to the formulation-induced structural changes of silk nanoparticles compared with air-dried silk films, showed values that ranged from 0.311 ± 0.021 to 0.441 ± 0.114 (**Figure 2.2f**).

Thioflavin T was also used as a model drug to determine the extent of solvent-accessible beta-sheet structure content, revealing that the percentage of model drug-loaded silk

nanoparticles detected by NTA ranged from $71\pm12\%$ to $76\pm10\%$ (**Figure 2.2g**). The fluorescence intensity measurements showed a similar drug content per particle for control and low Ca^{2+} mass ratio nanoparticles. However, the production yield was significantly improved resulting in 360% more particles (**Figure 2.3b**). A higher payload intensity was also observed for those silk nanoparticles formed with the 11.5 mg mass ratio of Ca^{2+} per 1 g of silk fibroin (**Figure 2.2h** and **Figure 2.3a**). The proposed mechanism on Ca^{2+} -modulated silk structural changes, incorporated in a liquid silk-silk nanoparticles transition, is illustrated in (**Figure 2.2i**).

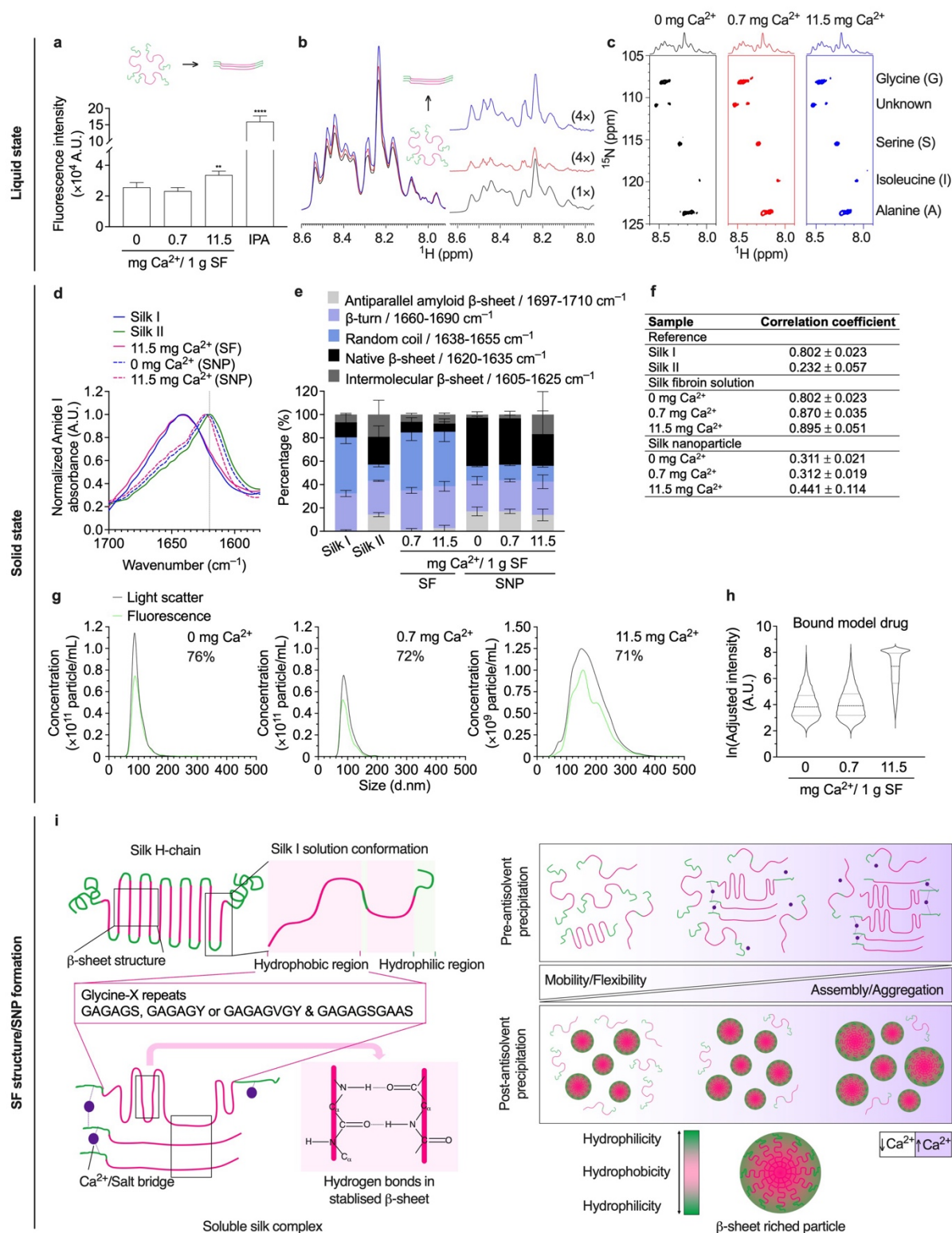


Figure 2.2 Structural and conformational analyses of silk solutions and nanoparticles. (a) Structural changes of aqueous silk fibroin (SF) in the presence of Ca^{2+} as assessed by thioflavin T assays (ThT); isopropanol (IPA) ($n=3$), (b) 1D ^1H -NMR, (c) and 2D [^1H , ^{15}N] HSQC NMR spectra in the NH region ($n=1$). The 1D ^1H NMR spectra (left); difference spectra (red and blue) compared with control spectrum (black) (right); all NMR data: black - control; red - with 0.7 mg Ca^{2+} (7% increase); blue - with 11.5 mg Ca^{2+} (17.5% increase). FTIR secondary structure analyses of control silk films with low (silk I) and high (silk II) β -sheet content and silk nanoparticles with (d) the normalized amide I absorbance spectra, (e) secondary structure content, (f) and correlation coefficient. (mean $\pm$ SD, $n=3$). (g) The overlay of model drug-loaded silk

nanoparticle concentration corresponds to size distribution measured by nanoparticle tracking analysis (NTA). The light scatter data are shown as black lines and the fluorescence data are shown as green lines for 1 mg/mL samples. **(h)** The fluorescence intensity of the payload detected by nanoparticle tracking. One-way ANOVA and Dunnett's multiple comparisons test were used for statistical analysis, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). **(i)** Schematic of the proposed Ca^{2+} -enhanced silk self-assembly process. All experiments were conducted as three independent experimental repeats, and with three technical repeats each for FTIR study ($n = 3$).

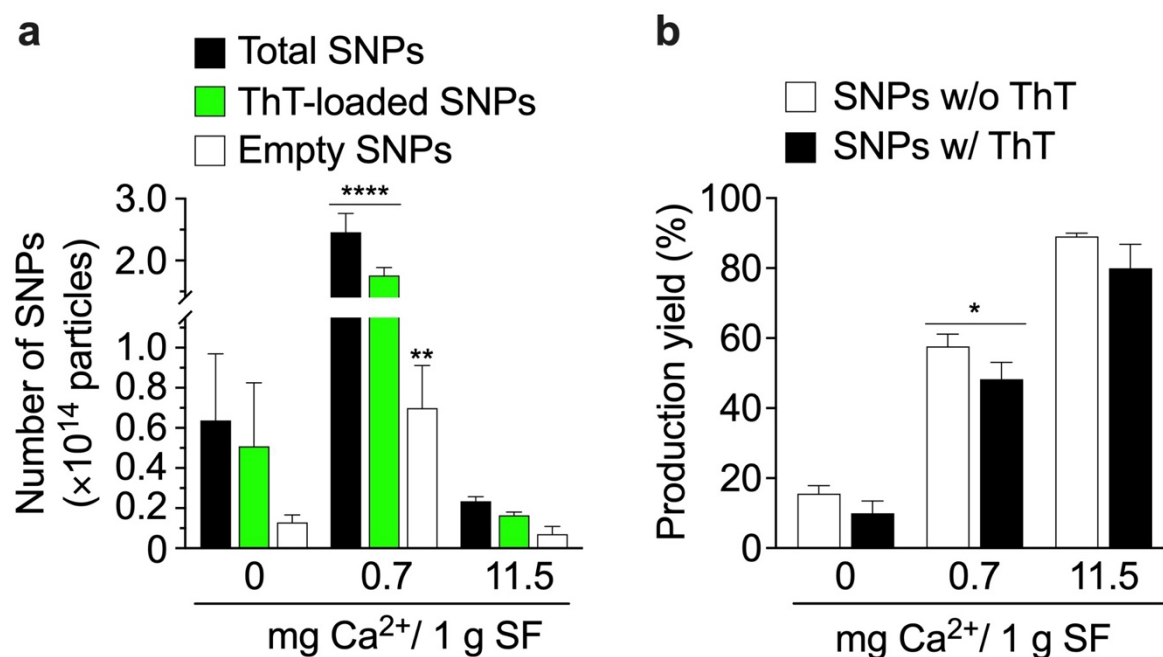


Figure 2.3 Thioflavin T-loaded silk nanoparticles (ThT-loaded SNP) manufactured in the 0–11.5 mg Ca^{2+} range. **(a)** Fraction of ThT-loaded and empty silk nanoparticles by NTA in scatter and fluorescence mode. **(b)** The production yield of silk nanoparticles obtained with and without ThT addition normalized to dried silk fibroin (SF) weight. The Two-way ANOVA and Šídák's multiple comparisons test were used for statistical analysis, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). All experiments were conducted as three independent experimental repeats ($n = 3$).

For FTIR analysis, two different IR fitting methods and peak assignments were performed in this thesis, revealing variations in the secondary structure analysis (**Figure 2.5** and **Figure 2.5**).

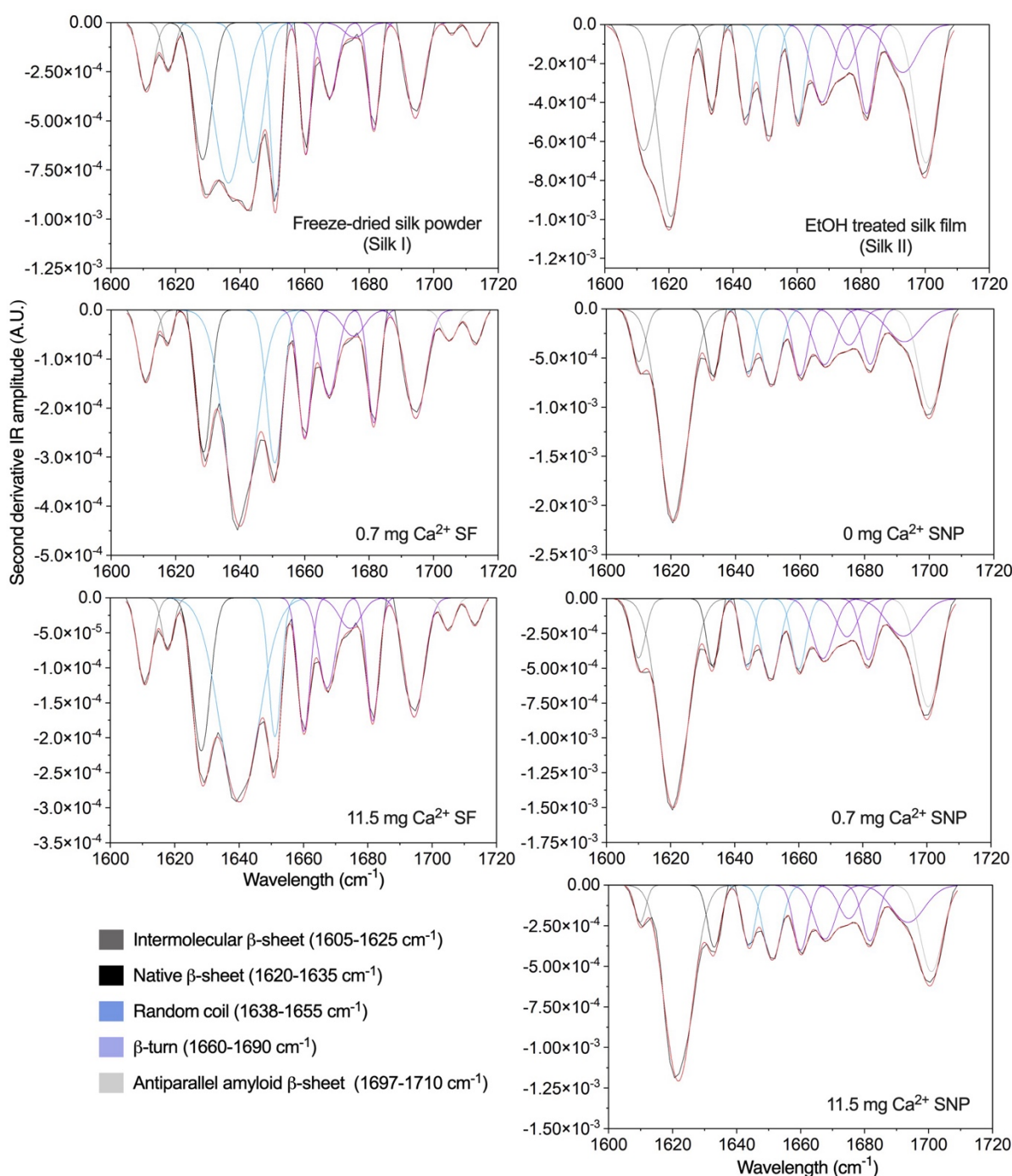


Figure 2.4 The exemplary IR fitting of the amide I region for Ca^{2+} -mixed silk fibroin (0.7 and 11.5 mg Ca^{2+} SF) and Ca^{2+} -mixed silk nanoparticle (0, 0.7 and 11.5 mg Ca^{2+} SNP) in comparison with the freeze-dried silk powder (silk I) and EtOH-treated silk film (silk II) control. The freeze-dried silk powder (silk I) serves as a 0 mg Ca^{2+} -mixed SF. All experiments were conducted as three independent experimental repeats with three technical repeats each ($n = 3$).

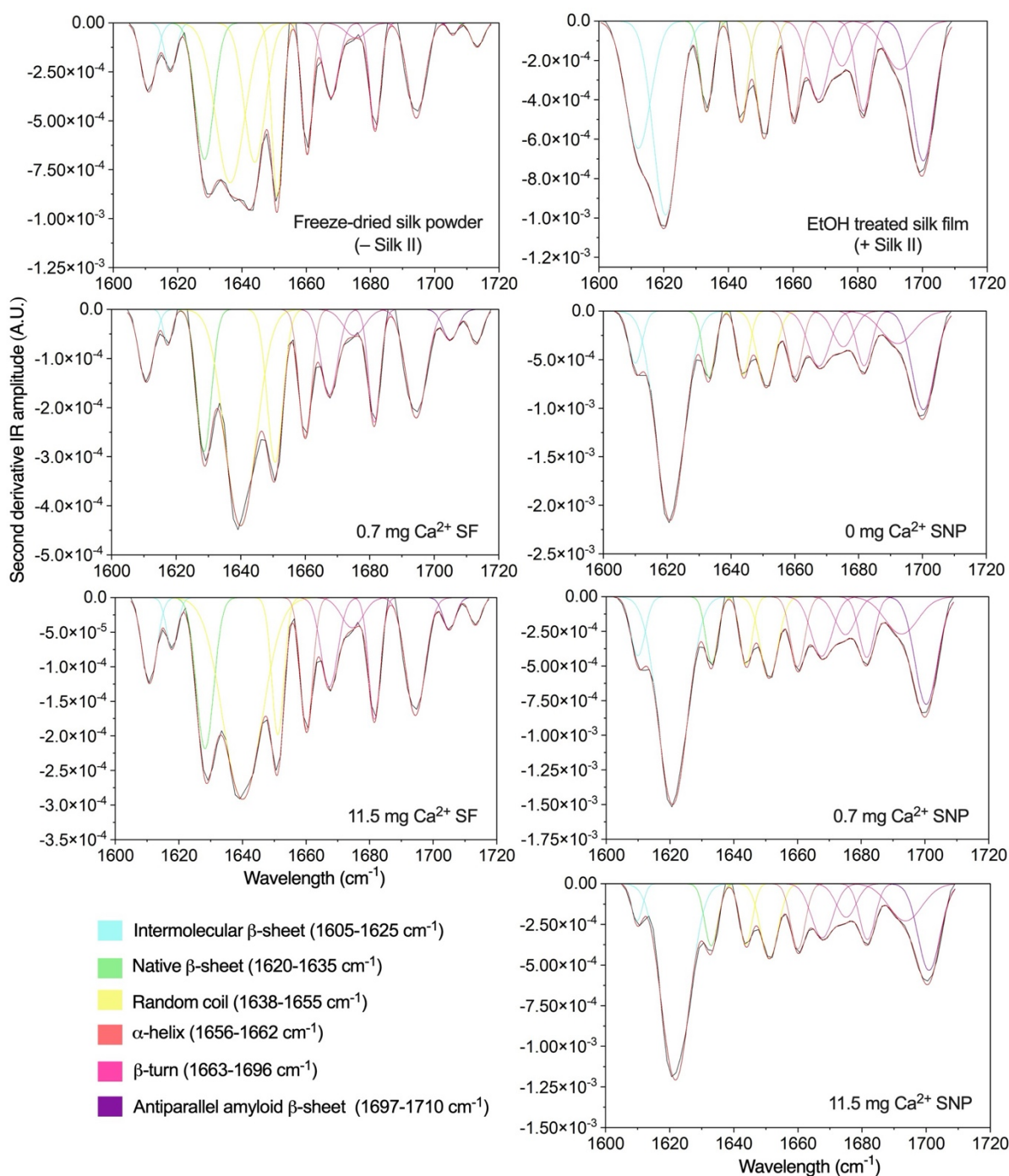


Figure 2.5 The exemplary IR fitting of the amide I region, including the peak assignment for α -helix, was performed for Ca²⁺-mixed silk fibroin (0.7 and 11.5 mg Ca²⁺ SF) and Ca²⁺-mixed silk nanoparticles (0, 0.7, and 11.5 mg Ca²⁺ SNP), in comparison with freeze-dried silk powder (silk I) and EtOH-treated silk film (silk II) controls. The freeze-dried silk powder (silk I) serves as the 0 mg Ca²⁺-mixed SF. All experiments were conducted as three independent experimental repeats ($n = 3$).

2.3.2. Particle Size and Morphology of Silk Nanoparticles

Silk nanoparticles were manufactured at a low (0.7 mg Ca^{2+}) and a high Ca^{2+} (11.5 mg Ca^{2+}) mass ratio to mimic the natural Ca^{2+} mass ratio per gram of silk fibroin reported in the middle silk gland as the highest experimental ratio. The impact of Ca^{2+} on silk nanoparticle physicochemical properties was determined using dynamic and electrophoretic light scattering. The increase in Ca^{2+} mass ratio significantly increased the particle size from 87.1 d.nm to 110.3 d.nm and 262.8 d.nm for low and high mass ratio Ca^{2+} , respectively. However, the samples maintained a low polydispersity index. The production yield was significantly increased from 16% to 89% in a Ca^{2+} concentration-dependent manner (**Figure 2.6a**).

The zeta potential value reflects the nanoparticle surface electrical charge, whereby a higher zeta potential indicates greater stability and less aggregation.⁵³ Ca^{2+} affected the zeta potential value of the silk nanoparticles, as a low mass ratio of Ca^{2+} raised the zeta potential value from -39.5 mV to -49.4 mV, while a high mass ratio of Ca^{2+} dropped the zeta potential value to -24.4 mV (**Figure 2.6a**).

I used field emission scanning electron microscopy to assess the morphology and size of dehydrated silk nanoparticles. All silk nanoparticles had a spherical morphology with coarse surface appearance at low Ca^{2+} mass ratios, but the surfaces became smooth at high Ca^{2+} mass ratios and a circularity of 0.8-0.9 was maintained. These images showed a consistent trend in particle size changes compared with the dynamic light scattering, increasing in size from 64 ($\pm$ 14) to 173 ($\pm$ 62) nm (**Figure 2.6b**). The proposed physicochemical properties and production yield of the resulting silk nanoparticles align with the characteristics of nanoparticles intended for drug delivery (detailed in **Table 2.1**).

Table 2.1 The proposed specification for SNP physicochemical properties and production yield.

Parameter	Referenced SNP ²³	Proposed SNP
Particle size (d.nm)	114	100–500 ¹⁹⁹
Polydispersity index (PDI)	0.14	≤0.2 ²⁰⁰
Zeta potential (mV)	–33	±30 or higher ^{201, 202}
Production yield (%)	9	>20

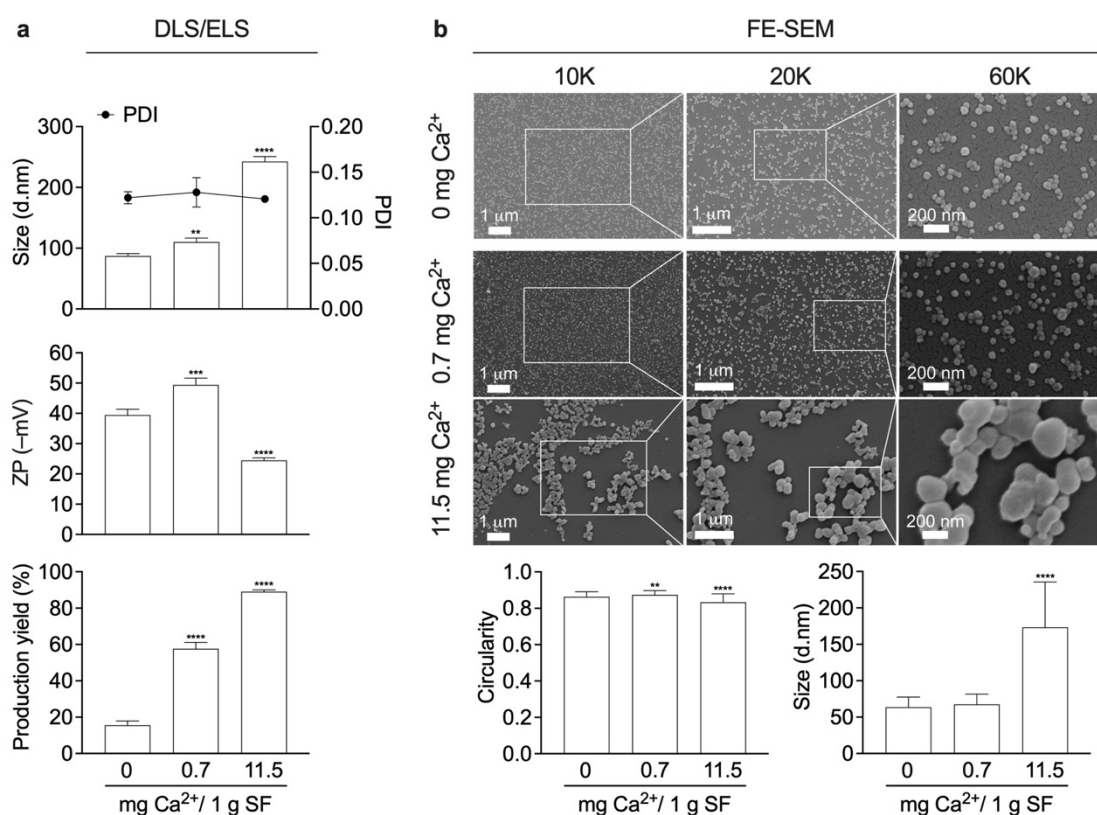


Figure 2.6 Silk nanoparticle characteristics. (a) Analysis of size (DLS), zeta potential (ZP) (ELS) and yield of silk nanoparticles ($n = 3$). **(b)** FE-SEM micrographs reported at 10K, 20K, and 60K magnifications. 400–500 particles and 100–150 particles derived from at least three different regions of interest were used to perform size and circularity calculations. Metal ion ratios were expressed as mg per 1 g of silk fibroin (SF). One-way ANOVA and Dunnett's multiple comparisons test were used for statistical analysis; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). DLS, ZP, and production yield were conducted as three independent formulation batches with three technical repeats each ($n = 3$). FE-SEM was conducted as one experimental repeat with three technical repeats for computed circularity and size analyses ($n = 1$). PDI: Polydispersity index

2.3.3. Cellular Response to Silk Nanoparticles *in vitro*

The biocompatibility of silk nanoparticles was tested using the RAW 264.7 murine macrophage cells. The low Ca^{2+} mass ratio-mixed silk nanoparticles exhibited a low cytotoxicity comparable to that observed for control silk nanoparticles, with a growth reduction of <15% at silk nanoparticle concentrations <250 $\mu\text{g/mL}$. Similarly, silk nanoparticles applied at 31.25–125 $\mu\text{g/mL}$ caused a growth reduction of <20%. Cell viability gradually decreased over the test concentration range; however, the IC_{50} remained higher than the maximum concentration (500 $\mu\text{g/mL}$) (**Figure 2.7a**).

Inflammatory responses to silk nanoparticles were elucidated by comparison to lipopolysaccharide-treated macrophages, which served as a positive control for inflammation. Inflammatory cytokines and chemokines in the cell culture media were measured after a 24 h nanoparticle exposure. The Ca^{2+} -mediated silk nanoparticle noticeably upregulated interleukin-1 receptor antagonist (IL-1ra), monocyte chemoattractant protein-1 (MCP-1/CCL2/JE), macrophage inflammatory protein-1 α (MIP-1 α), and macrophage inflammatory protein-1 β (MIP-1 β), whereas interferon gamma-induced protein 10 (IP-10) was downregulated (**Figure 2.7b** and **Figure 2.8**). All tested silk nanoparticles induced negligible amounts of endogenous nitrite (NO_2^-) when compared with the positive control (4.9 μM). The $\text{TNF-}\alpha$ levels (<0.07 ng/mL) showed no significant differences when comparing baseline and silk nanoparticle groups (**Figure 2.7c**).

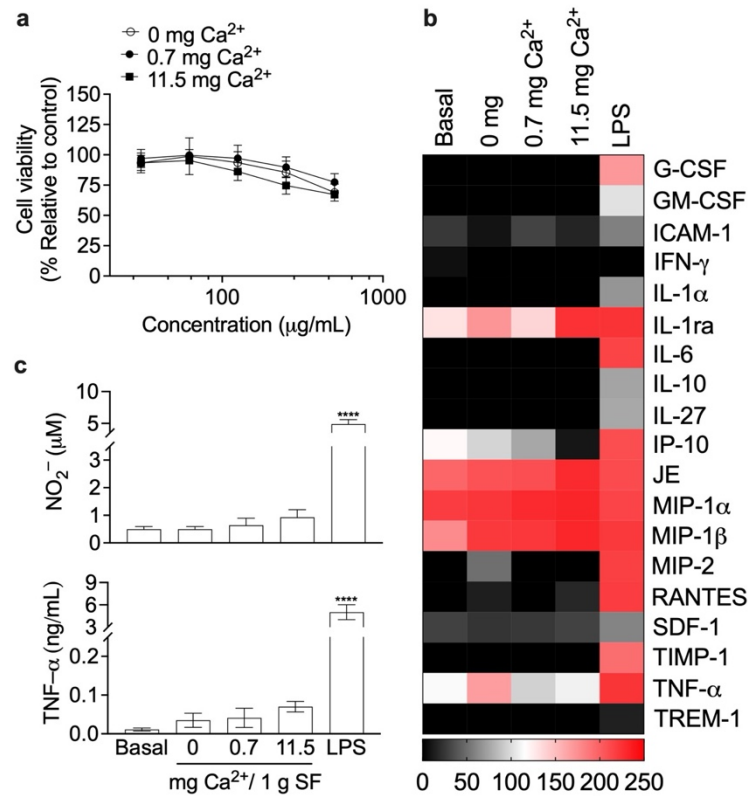


Figure 2.7 Impact of silk nanoparticles on RAW 264.7 murine macrophages. (a) Cell viability and inflammatory responses; (b) inflammatory cytokine expression level, (c) endogenous nitrite (NO_2^-) and $\text{TNF-}\alpha$ levels in response to the silk nanoparticles. Macrophages treated with complete media supplemented with 200 ng/mL lipopolysaccharide (LPS) served as an inflammation-positive control. One-way ANOVA and Dunnett's multiple comparisons test were used for statistical analysis, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). All cell-based assays were conducted as three independent cell experiment repeats ($n = 3$).

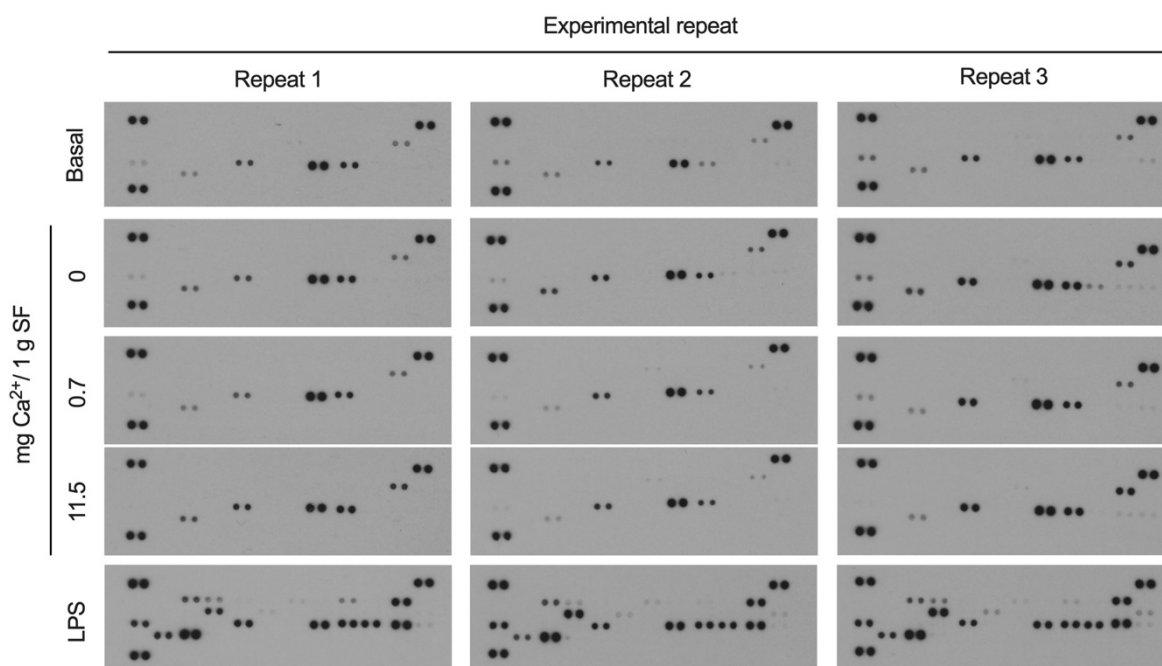


Figure 2.8 Inflammatory cytokine detection in RAW 264.7 murine macrophages in response to silk nanoparticle exposure; 500 $\mu\text{g/mL}$. Expressed cytokines obtained from three experimental repeats. Cells were treated with complete media supplemented with 200 ng/mL lipopolysaccharide for positive control. Experiment was conducted as three independent cell experiment repeats ($n = 3$).

2.4. Discussion

2.4.1. Impact of Ca^{2+} on the Secondary Structure of Aqueous Silk and Silk

Nanoparticles

Protein-based nanoparticles are commonly prepared by emulsification,⁷⁶ complex coacervation,⁷⁸ nanospray drying,²⁰³ electrospraying,⁸⁰ desolvation,¹⁶⁴ and self-assembly²⁰⁴. Yet, the ability to control particle size, achieve yields of $> 80\%$, and capitalize on biomimetic principles remains an unmet challenge. This study produced silk nanoparticles by antisolvent nanoprecipitation using isopropanol to induce silk self-assembly with a core β -sheet rich nanoparticle with a submicron size. In the presence of IPA, surface charge disruption and inter- and intramolecular interactions dehydrate protein molecules to drive particle formation.²⁰⁵ The pH, ionic strength, desolvating agent, crosslinking agent and encapsulated drug content

typically govern the size and physicochemical properties of resulting nanoparticles.^{190, 197} This study revealed that combining Ca^{2+} and isopropanol maximized yields, modulated and tuned the size and physicochemical properties of the silk nanoparticles. The Ca^{2+} to silk mass ratio used was based on the ratio reported in the silk gland.¹²

The impact of Ca^{2+} on the structural transformation of silk was elucidated by FTIR analysis to compare the secondary structure content of the silk nanoparticles and precursor materials (i.e., the Ca^{2+} -mixed silk solution). Seminal work by Tetsuo Asakura and colleagues, using NMR demonstrated that native silk, prior to spinning, adopts a predominantly β -turn structure.²¹ This foundational research has informed my FTIR analysis to monitor relative changes in secondary structure (**Figure 2.2e**). Peaks occurring in the $1656\text{--}1662\text{ cm}^{-1}$ region were attributed as being α -helical content²⁰⁶⁻²⁰⁸ (**Figure 2.5**),^{23, 209, 210} enabling cross-study comparisons with the literature. FTIR analyses were supplemented with NMR and Thioflavin T analysis to strengthen my conclusions. The silk I and silk II references exhibited a typical amide I region, which shifted from 1640 cm^{-1} to 1620 cm^{-1} . The spectra of Ca^{2+} -mediated and control silk nanoparticles were consistent with the crystalline silk II reference, indicating an increase in β -sheet structure (**Figure 2.2d**).

In the presence of Ca^{2+} , the transient salt bridges formed between Ca^{2+} and an estimated 77 carboxylate-substituted amino acids (aspartic acid and glutamic acid) in silk contribute to a strong inter- and intramolecular interaction between silk chains.²¹¹ These salt bridges stabilize the silk formation network, resulting in increased viscosity and the creation of a gel-like liquid state.¹⁷ Thus, pre-incubating a reverse-engineered aqueous silk with Ca^{2+} may cause the formation of a chelating network, thereby yielding a higher order of stabilized β -sheets along with an increase in the Ca^{2+} mass ratio.^{18, 28} The Ca^{2+} ions therefore induced a liquid crystalline-like state.³³ The metal ion valence state has also been reported to affect the self-assembly behaviour of silk, whereby Ca^{2+} at 100 positive charges per silk chain can

accelerate the formation of a hydrodynamic diameter (d_H) that reduces diffusion and creates a stronger interaction between silk chains.²¹¹

Antisolvent nanoprecipitation in isopropanol using a semi-batch format induced structural changes in which the hydrophilic blocks of the H-chain had a low solubility that promoted the self-assembly of β -sheet-rich silk nanoparticles.²³ FTIR analysis showed a conformational transition during silk nanoparticle synthesis, as evidenced by the higher β -sheet content of the silk nanoparticles compared to the silk film control. However, in the presence and absence of Ca^{2+} the overall degree of β -sheet content in the respective silk nanoparticles were comparable. This result demonstrates that Ca^{2+} does not significantly affect overall secondary structure changes; however, a high mass ratio Ca^{2+} noticeably increases the proportion of intermolecular β -sheet structure. I speculate that Ca^{2+} induced a liquid crystalline-like state by influencing silk stability and fluidity.

This study also revealed significant fluorescence intensity of bound thioflavin T in the presence of a high Ca^{2+} mass ratio. Thioflavin T is a fluorescent dye consisting of benzothiazole and dimethylaminobenzene. Changes in rotation of these aromatic rings, derived from the binding or through intercalation, alter the fluorescence emission intensity.²¹² Thioflavin T serves as a gold standard for detecting amyloid binding and formation,^{213, 214} β -sheet rich protein aggregation,^{215, 216} and nucleic acid binding and recognition.^{212, 217} The thioflavin T findings reported here supported the notion that Ca^{2+} promotes silk self-assembly and increases β -sheet content in the aqueous phase.

NTA analysis of the ThT-labelled silk nanoparticles showed that Ca^{2+} not only increased particle yield and size but also significantly increased drug loading. I hypothesize that Ca^{2+} may enhance both silk-silk interaction and payload-silk interaction, resulting in higher payload content in silk nanoparticles at increased Ca^{2+} mass ratios in parallel with size tuning (**Figure 2.2g**, **Figure 2.2h**, and **Figure 2.3**).

Conformational changes in silk structure were further explored by 1D ^1H NMR and 2D [^1H , ^{15}N] HSQC NMR analysis. Notable increases in signal area and intensity were recorded for the NH resonance region of the 1D ^1H NMR data with increasing Ca^{2+} content. This NMR signal increase implies that a greater proportion of all the labile hydrogens occupied the same local NH chemical environment, indicating an alteration of protein stability (i.e., denaturation, fragmentation, and self-assembly).²¹⁸ This study showed that increasing the Ca^{2+} concentration increased the area of the NH region, suggesting a change in the on/off exchange rate of the labile NH protons. If I consider that the increase in signal correlates with a reduced rate of exchange, then the NH protons spend more of their time attached to nitrogen. One implication of this could be a greater degree of a liquid crystalline-like state, which could subsequently aid silk nanoparticle formation.

These NMR studies enabled us to monitor the molecular mobility, and these data suggest a greater degree of self-assembly at the onset of the assembly into larger particles.²¹⁸
²¹⁹ This hypothesis is supported by earlier evidence showing that metal ions influenced the folding characteristics of silk fibroin, as indicated by an increase in the ^{13}C CP-MAS NMR spectra intensity in the alanyl C_β region (22–12 ppm) and the transitioning of the silk from an α -helix to a β -sheet conformation.¹¹⁰ The most abundant amino acids in the silk heavy chain are glycine, alanine and serine, and the 2D [^1H , ^{15}N] HSQC solution NMR data showed that their respective signals displayed random coil chemical shifts that are consistent with a mobile, "unstructured" silk molecule (i.e. no β -sheets) although these hydrophobic amino acid motives are ultimately implicated in β -sheet formation and silk mechanics.^{2, 24}

Silk nanoparticle formation occurs when silk is combined with an anti-solvent, resulting in supersaturation and the nucleation of particles through Gibbs free-energy self-assembly mechanism.²²⁰ During this process, collisions between silk chains form complexes that develop into stable nuclei, and as protein concentration decreases, growth in larger nuclei

is enabled.^{196, 220} Overall, these findings confirmed the impact of Ca^{2+} on silk conformation and stability. This effect arose due to the formation of salt bridges between Ca^{2+} and acidic amino acids that then caused a strong attractive force between silk protein chains,^{17, 18, 21, 28} reduced the number of unfolded silk chains, and yielded silk soluble complexes. This phenomenon enhanced silk folding and a conformational transition that favoured β -sheet structure^{110, 193} and created a better hydrogen bonding geometry of C=O groups and/or NH groups in the β -sheet conformation.²²¹ Therefore, I speculate that the local environment of key amino acid residues was more stabilized. These stabilized silk complexes enabled the silk assembly in isopropanol to proceed because the overall entropy in the system was reduced through Ca^{2+} -mediated pre-ordering of the silk structure into a liquid crystalline-like state. This, in turn, reduced the ‘activation barrier’ to silk nanoparticle formation, making it ‘easier’ for silk nanoparticles to grow (and for gel formation to occur more readily) (**Figure 2.2i**). The high Ca^{2+} mass ratio resulted in a significant difference in bound thioflavin T fluorescence intensity and a dramatic difference in ^1H NMR spectra. Taken together, these findings confirmed that Ca^{2+} significantly impacts the silk nanoparticle size-tuning process in a Ca^{2+} concentration-dependent manner *via* ion coordination, which enhances liquid-crystalline transition and/or structural ordering.

5.3.8. Impact of Ca^{2+} on Particle Size and Morphology of Silk Nanoparticles

Particle size increased with ascending Ca^{2+} concentrations. These findings are supported by an orthogonal study by Ki Hoon Lee and co-workers, who showed that Ca^{2+} concentrations influenced silk-dextran liquid-liquid phase separation, thereby affecting coacervate size and turbidity of the solutions.²²² The corresponding phase separation map²²² of silk-dextran in response to Ca^{2+} ion concentration resulted in a one-phase and two-phase system at the corresponding low and high Ca^{2+} ion concentrations used in the present work. However, the absence of a crowding agent in my study negates the liquid-liquid phase separation phenomenon.

Morphological assessment by electron microscopy confirmed spherical topology of the silk nanoparticles, but the size was smaller compared with DLS measurements (**Figure 2.6**). This discrepancy can be expected due to different analysis modes and sample states. Corroborating this, larger particles showed a greater extent of shrinkage (27–40%) that in turn implies greater swelling capacity in solution.

Increasing the Ca^{2+} mass ratio was also equivalent to increasing the concentration of counter ion (Cl^-) in a desolvating system altering the ionic balance of silk nanoparticles and their net charge leading to a higher zeta potential for a low mass ratio of Ca^{2+} , but a lower zeta potential for a high mass ratio of Ca^{2+} . The high Ca^{2+} mass ratio might indicate a saturation of ion-mediated electrostatic shielding to specific charge interaction of both Ca^{2+} and Cl^- on silk protein.²²³ Due to the dominant forces in protein folding, especially a hydrophobic interaction occurs during a silk assembly and silk nanoparticle formation, the polar/charged residues will be exposed on silk nanoparticle surface protecting a rigid cored structure (β -sheet).²²³ This changed the net charge surface of silk nanoparticles, resulting in a decreased zeta potential index. By contrast, no change was observed in the polydispersity index, indicating a dispersion appropriate for nanomedicines ($\text{PDI} < 0.2$).^{200, 224}

2.4.3. Impact of Silk Nanoparticles on Macrophages

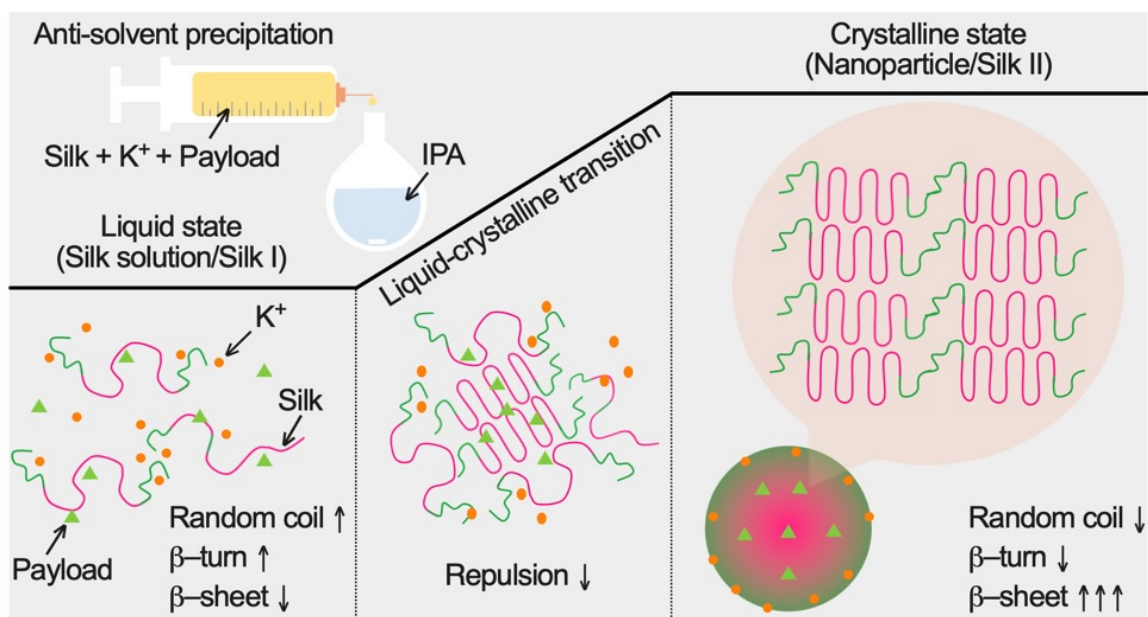
In this study, the cytotoxicity and inflammatory response following silk nanoparticle exposure were evaluated in a macrophage model to profile nanoparticle biocompatibility. Monitoring macrophage health, to gauge biocompatibility, is useful because macrophages serve as a key first-line defence mechanism.^{225, 226} Nanoparticles have recently been implicated in alterations of the macrophage phenotype, including induction of polarization and reprogramming and modulation of pro- and anti-inflammatory cytokines and chemokines.²²⁷⁻²²⁹ However, treatment of macrophages with any of the tested silk nanoparticles resulted in no significant upregulation of endogenous NO_2^- and $\text{TNF-}\alpha$.

Particle size-dependent cellular responses are described in literature; for example, a greater degree of inflammation was induced by submicron amorphous silica particles (30–1000 d.nm) than by particles >1000 nm,²³⁰ while a higher level of cytotoxicity, inflammation, genotoxicity and developmental toxicity was promoted by small silver nanoparticles (20 d.nm) than by larger particles (80 and 113 d.nm).²³¹ The effects of any silk nanoparticles on macrophages revealed negligible variations in JE or MIP-1 α , an upregulation of IL-1 α and MIP-1 β , and a downregulation of IP-10 compared with baseline levels. Here, none of the tested silk nanoparticles promoted an excessive production of pro-inflammatory cytokines (IL-1 α , IL-6, G-CSF, GM-CSF, and TIMP-1),²³²⁻²³⁴ an anti-inflammatory cytokine (IL-10)²³⁵ or a pro-inflammatory chemokine (TREM-1)²³⁶ compared with lipopolysaccharide-activated macrophages. In summary, silk nanoparticles exhibited low acute toxicity and no overt LPS-like activation in RAW 264.7 cells under the tested conditions, supporting a first-pass screening suitable for drug delivery

2.5. Conclusion

This study used Ca²⁺ ion spiking of liquid silk to coordinate silk solution properties and to promote silk nanoparticle self-assembly using antisolvent nanoprecipitation in a semi-batch format. Successful improvement of the physicochemical properties and scalability of silk nanoparticles was achieved by mimicking the natural function of Ca²⁺ in silk processing by silkworms. The Ca²⁺: silk mass ratio of storage silk in the middle silk gland was selected as my high Ca²⁺ condition and was compared to a 16-fold lower condition (0.7 mg/g). The findings confirmed the impact of Ca²⁺ on silk conformational and structural properties. In the presence of Ca²⁺, I observed the formation of liquid silk crystals that transitioned to solid β -sheet rich silk nanoparticles. These nanoparticles showed good biocompatibility in the macrophage model.

Chapter 3 Optimizing Silk Nanoparticle Assembly with Potassium Ions: Effects on Physicochemical Properties and Encapsulation Efficiency



Chapter 3 details the results from the published article in ACS Applied Bio Materials (Roamcharern, N.; Brady, D. J.; Parkinson, J. A.; Rattray, Z.; Seib, F. P. Optimizing Silk Nanoparticle Assembly with Potassium Ions: Effects on Physicochemical Properties and Encapsulation Efficiency, ACS Applied Bio Materials, 2025, 8(8), 6854-6864. (DOI: <https://doi.org/10.1021/acsabm.5c00598>). This work was carried out concurrently with Chapter 2, focusing on the influence of potassium ions (K⁺) on the physicochemical properties of silk nanoparticles. The manufacturing process was optimized, including the refinement of the potassium ion (K⁺) mass ratio used in nanoparticle synthesis. I was responsible for designing and conducting experiments involved in nanoparticle characterization. In collaboration with Dr. John Parkinson and Dr. Daniel Brady, I conducted an NMR study using isotope-labeled silk cocoons prepared by Dr. Brady. The experimental design and data interpretation for the NMR analysis were carried out with guidance from Dr. Parkinson. This chapter was completed under the supervision of Prof. Zahra Rattray and Prof. Philipp Seib.*

3.1. Introduction

Silk fibroin from the *Bombyx mori* silkworm is recognized as a natural biomaterial with favorable mechanical strength, biocompatibility, and biodegradability^{24, 188-190} and with a broad spectrum of formats available through various processing protocols.^{237, 238} Silk fibroin-based nanoparticles have been widely fabricated using regenerated aqueous silk fibroin extracted and dissolved from silk cocoons.^{89, 131, 175} Recently, several techniques have been developed for nanoparticle manufacture from liquid fibroin, with anti-solvent precipitation confirmed to be a highly effective method with convenient operation, no requirement for expensive apparatus, and desirable time efficiency.^{2, 75, 82, 197} The antisolvent process involves first dissolving a biopolymer in a liquid in which the material has high solubility, followed by gradual addition of an anti-solvent, a liquid in which the biopolymer has low solubility. The anti-solvent precipitation approach enhances nucleation, growth, and agglomeration of the biopolymer in the mixed solvent–anti-solvent system.⁸⁵

The *B. mori* silk protein structure consists of a light chain (L-chain, ~26 kDa) linked to the silk heavy chain (H-chain, ~391 kDa). The heavy chain has a nonrepetitive amino acid sequence located at the C- and N-termini and a unique repeating amino acid sequence of hydrophobic and hydrophilic blocks.¹¹ The hydrophobic blocks contain repetitive glycine-X repeats accounting for 94% of H-chain, with X mostly being alanine (A) (65%), serine (S) (23%), or tyrosine (Y) (9%) by composition.² The overall silk H-chain structure is presented as an anti-parallel β -sheet structure, stabilized by hydrogen bonds, Van der Waals forces, and hydrophobic interactions; therefore, altering the ordered β -sheet degree simply impacts the silk mechanical properties.^{2, 192, 239}

Liquid silk is affected by various factors, such as pH,³² metal ions,^{17, 240, 241} shear flow,^{29, 242} temperature,²⁴³ and solvents,²⁴⁴ which can influence its conformation. Metal ions are a major factor that influences the structure of the liquid silk in the silk gland and play an

important role in spinning of the silk fiber. Our previous study highlighted the significant role played by Ca^{2+} (divalent ion), a key modulator of silk conformation and structure in the silk gland, in the fabrication of silk nanoparticles.²⁴⁵ However, changes occur in the conformation and structure of silk during processing due to various factors, notably due to the presence of various metal ions, including K^+ (monovalent ion), throughout the silk gland.²¹¹

In nanoparticle formation, the counterion facilitates peptide self-assembly through ionic interactions, with its Debye length influencing whether it preferentially interacts with peptides or water molecules. Ion concentration and solution ionic strength affect the Debye length, thereby regulating self-assembly and particle size *via* this molecular mechanism, by allowing peptides to come into closer proximity.^{246, 247} Divalent and monovalent ions influence the Debye sphere differently, thereby affecting electrostatic interactions in solution. Divalent ions tend to promote more compact assemblies, while monovalent ions result in less impact assemblies.²⁴⁸ The aim of the present study was to unravel the effects of K^+ on silk structure because K^+ abundance in the silk gland is comparable to that of Ca^{2+} during the storage and spinning processes. In addition, the K^+ ratio increases significantly from about 3000 $\mu\text{g/g}$ dry weight to 10000 $\mu\text{g/g}$ dry weight in the gland luminal contents and it regulates the silk conformation and stability.^{17, 28}

Here, I investigated the potential impact of K^+ on silk processing within my silk nanoparticle manufacturing system that utilizes anti-solvent precipitation in a semi-batch format. I expected the resulting nanoparticles to display distinct physicochemical properties and favorable characteristics for functioning as nanodrug carriers, particularly after tuning the silk processing protocol for nanoparticle fabrication at the laboratory scale. I also examined the β -sheet-rich structure of silk nanoparticles, as this could drive potential future applications in pharmaceutical design for a variety of hydrophobic drugs and biomolecule loading.²⁴⁹ Additionally, I used Thioflavin T as a model hydrophobic drug to examine silk behavior in both

liquid form and as a solid nanoparticle, and to assess the impact of K⁺ on silk assembly and encapsulation efficiency. I predict that the findings presented here will provide a development strategy for improving silk-based nanocarrier systems.

3.2. Materials and Methods

3.2.1. Materials

All reagents and solvents were purchased from Sigma-Aldrich or Acros Organics (>98% purity) and used without additional purification unless noted. *B. mori* silk cocoons were purchased from Scientific Center on Sericulture, Vratsa, Bulgaria.

3.2.2. Aqueous Silk Preparation

Aqueous silk fibroin was prepared using *B. mori* silk cocoons, following a protocol described elsewhere.²⁴⁵ The silk cocoons were chopped into small pieces (5×5 mm) after removing contaminants (e.g., soil and dust). A 5 g sample of cocoon pieces was degummed in 2 L of 0.02 M Na₂CO₃ by boiling for 1 h under gentle stirring. The degummed silk was washed three times in 1 L of deionized water for 20 min, squeezed to remove excess water, and then dried overnight at ambient temperature in a fume hood.

The dried degummed silk was dissolved in 9.3 M LiBr with a silk:LiBr ratio of 1 g to 4 mL. After incubation at 60 °C for 4 h, the resulting aqueous silk solution was dialyzed against deionized water (Slide-A-Lyzer 3.5K Dialysis Cassette G2, Thermo Scientific, Rockford, IL, USA) for 48 h. The aqueous liquid silk preparation was centrifuged three times at 2,885g for 40 min at 4 °C (PK 121R Centrifuge, rotor T515, ACL International Srl, Milan, Italy), and the supernatant was transferred to a fresh container and kept at 4 °C until further analysis. The concentration of the aqueous silk solution (% w/v) was calculated as follows.

$$\% w/v = \left[\frac{(W2-W1)}{0.5} \right] \times 100 \quad \text{Equation 3.1}$$

where, W1 is the weight of empty weighing boat (g) and W2 is the weight of the weighing boat containing 0.5 mL silk fibroin after drying at 60 °C for at least 72 h.

3.2.3. Silk Nanoparticle Manufacture

Silk nanoparticles were synthesized using the anti-solvent precipitation method in a semi-batch format with previously-described optimized parameters.²⁴⁵ The potassium ion (K⁺)-induced silk nanoparticles were fabricated at ambient temperature. Briefly, 3% w/v aqueous silk was supplemented with KCl to give mass ratios of K⁺ (mg) to silk (g) of 0, 1.1, and 17.3 mg/g. The highest mass ratio mimicked the K⁺ to silk content in the middle silk gland, while a 2-fold dilution to the lowest ratio was used to assess concentration-dependent size tuning. The resulting aqueous silk mixtures were added dropwise from a height of 7.5 cm above the meniscus to 30 mL of isopropanol (IPA) using a syringe-pump speed of 1 mL/min and a stirring speed of 400 rpm. The silk nanoparticle suspension was centrifuged at 48,400g at 4 °C for 2 h, followed by sonication in deionized water. The centrifugation and sonication cycles were repeated twice more, and the final silk nanoparticle product was sonicated and dispersed in deionized water. All silk nanoparticle suspensions were produced in triplicate using different silk batches ($n = 3$).

3.2.4. Production Yield Determination

The silk nanoparticle production yield was calculated with **Equation 3.2**.

$$\% \text{ Yield} = \frac{\text{Concentration of silk nanoparticle suspension (\% w/w)} \times \text{Mass (g)}}{\text{Concentration of aqueous silk (\% w/v)} \times \text{Volume (mL)}} \times 100 \quad \text{Equation 3.2}$$

where, mass is the total weight of the silk nanoparticle product (g), while concentration of silk solution and volume are initial concentration (3% w/v) and initial volume (6 mL) used in anti-solvent precipitation, respectively. The % w/w was calculated as detailed in **Equation 3.3**:

$$\% \text{ w/w} = \left[\frac{(W3-W1)}{(W2-W1)} \right] \times 100 \quad \text{Equation 3.3}$$

where, W1 is the weight of empty weighing boat (mg), W2 is the weight of weighing boat containing 0.3 mL silk nanoparticle suspension (mg), and W3 is the weight of weighing boat containing 0.3 mL silk nanoparticle suspension after drying at 60 °C for ≥ 72 h (mg).

3.2.5. Thioflavin T Fluorescence Measurement of Aqueous Silk

The K⁺-induced silk fibroin structural changes in solution were studied with a Thioflavin T assay. First, 25 μ L of the silk fibroin mixtures (3% w/v silk; 0, 1.1, and 17.3 mg K⁺/ 1 g silk) were incubated with 25 μ L of 100 μ M Thioflavin T and 50 μ L of deionized water. A 60% v/v isopropanol sample served as a β -sheet rich (silk II) control. The mixtures were incubated at ambient temperature for 10 min in the dark, and then the bound Thioflavin T was measured (excitation = 440 nm, emission = 475 nm) using a fluorescence microplate reader (Polarstar Omega, BMG Labtech, Ortenberg, Germany).

3.2.6. Nuclear Magnetic Resonance Spectroscopy

For specific studies, *B. mori* larvae, raised on a 40% artificial diet (CREA, Sericulture Laboratory of Padua, Italy) under controlled conditions of 25 °C $\pm$ 1 °C and 75% relative humidity $\pm$ 5%, were used to produce modified isotope-labeled silk cocoons. In brief, 50 mg of D-glucose-¹³C6 (Cortec, Les Ulis, France) was added to 250 mg of dry artificial diet, and this labeled mixture was fed to the larvae twice daily during days 4–6 of the fifth instar. The larvae were then left to spin their cocoons.

Aqueous silk solution was produced, as detailed above, by adding the modified isotope-labeled silk to unmodified silk at a 1.5:1 protein mass ratio. A 0.5 mL sample of 3% w/v silk containing 0, 1.1, and 17.3 mg K⁺ per 1 g silk was prepared in deionized water. NMR spectroscopy was carried out by a Bruker 600 MHz AVANCE II⁺ NMR spectrometer following a previously reported method.²⁴⁵

3.2.7. Fourier-Transform Infrared Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was processed to investigate the secondary structure content of the liquid silk and nanoparticles. Freeze-dried silk and silk nanoparticles were prepared by freezing at $-20\text{ }^{\circ}\text{C}$ overnight, followed by lyophilization at $-10\text{ }^{\circ}\text{C}$ and 0.140 mbar for 24 h. Freeze-dried silk and 70% ethanol-treated silk films served as silk I and silk II references, respectively. The FTIR spectra were recorded over a wavenumber range of $400\text{--}4000\text{ cm}^{-1}$ with a resolution set at 4 cm^{-1} and scanning times set at 32 and 128 scans for background and sample channel scans, respectively (ATR-equipped TENSOR II FTIR spectrometer, Bruker Optik GmbH, Ettlingen, Germany). The secondary structure was analyzed by deconvoluting the FTIR amide I region ($1600\text{--}1700\text{ cm}^{-1}$). The wavenumber assignment for the secondary structure component were $1697\text{--}1710\text{ cm}^{-1}$, $1620\text{--}1635\text{ cm}^{-1}$, and $1605\text{--}1625\text{ cm}^{-1}$ for the total β -sheet structure, $1660\text{--}1690\text{ cm}^{-1}$ for the β -turn structure²¹ and $1638\text{--}1655\text{ cm}^{-1}$ for the random coil structure. The correlation coefficient (R) was calculated using second-derivative amide I spectra in comparison to an air-dried silk film, as outlined in a previous study.^{23, 245}

3.2.8. Particle Size Measurement of Silk Nanoparticles

Dynamic light scattering (DLS) was used to determine particle size, while nanoparticle tracking analysis (NTA) was employed to measure both particle size and concentration. DLS was carried out on 1:25 (v/v) ratio mixtures of 1 mg/mL silk nanoparticle suspension in deionized water. Analysis was performed under the following conditions: a count rate of 203.6 kcps, a measurement time of 70 s, a measurement position of 3 mm, and a temperature set at $25\text{ }^{\circ}\text{C}$ (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK).

Particle size and particle concentration (particle/mL) were studied with NTA (NTA 3.4 Build 3.4.003, Malvern Panalytical Ltd., Worcestershire, UK) at $25\text{ }^{\circ}\text{C}$. Briefly, the silk

nanoparticle suspension was diluted in sterile-filtered Milli-Q water to achieve a particle count of 20–80 particles per frame. The setup included an sCMOS camera, a 488 nm laser, a syringe pump set to a speed of 100 (Harvard Apparatus Model 98-4730 Syringe Pump, Massachusetts, US), and a viscosity of 1.0000 cP.

NTA data were recorded over five replicate 60 s videos using the light-scattering mode. The capture settings were optimized for silk nanoparticle characteristics, with a screen gain of 3 and camera level of 9 for smaller particle samples (0 mg K⁺ and 1.1 mg K⁺), and a screen gain of 1 and camera level of 8 for larger particle samples (17.3 mg K⁺). The data were analyzed using NTA 3.4 (version 3.4.003 software), with a detection threshold set at 6. Each silk nanoparticle type was analyzed in triplicate, with five technical replicates per replicate.²⁵⁰

3.2.9. Zeta Potential Measurement of Silk Nanoparticles

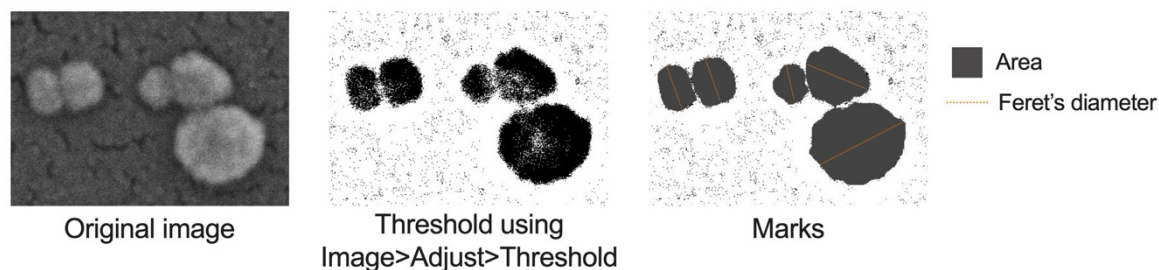
Zeta potential was measured using electrophoretic light scattering (ELS). Briefly, 5 mg/mL of silk nanoparticle suspension was diluted to a ratio of 1:20 (v/v) nanoparticles to deionized water using the following settings: temperature, 25 °C; count rate, 61.5 kcps; zeta run, 12; and measurement position, 2 mm (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK).

3.2.10. Analysis of Silk Nanoparticle Morphology

Silk nanoparticle suspensions were prepared at a concentration of 1 mg/mL in deionized water. A 20 µL aliquot of the suspension was applied to a 5 × 5 mm silicon wafer (Ted Pella, Inc., CA, USA), left to dry at ambient temperature for 24 h, and gold-coated by sputtering for 40 s from a 35 mm height with a pressure of 0.08 mb and current of 30 mA (Agar Scientific Manual Sputter Coater, Agar Scientific Ltd., Essex, UK). Silk nanoparticles were then observed using field emission scanning electron microscopy (FE-SEM) with a 5 kV voltage at magnifications of 10,000×, 20,000×, and 60,000× (Hitachi SU6600, Hitachi High-Tech Europe GmbH,

Krefeld, Germany). Circularity and size of silk nanoparticles were analyzed using Image J (NIH National Institute of Mental Health, Bethesda, MD, USA) (**Figure 3.1**).

a Image processing using Image J



b Analyze > Set measurement > Measure

Interested parameters	Equation/Definition	Interpretation
Circularity	$\frac{4\pi \times \text{area}}{\text{Perimeter}^2}$	1.0 = Perfect circle 0.0 = Elongated shape
Feret's diameter	The longest distance between any two points along the selection boundary.	Longer = Larger particle size Shorter = Smaller particle size

Figure 3.1 Schematic of silk nanoparticle circularity and size assessment using ImageJ software. (a) Image processing: threshold setting and particle boundary selection. **(b)** Circularity and Feret's diameter are analyzed by the function of Analyze, Set measurement, and Measure.

3.2.11. Model Drug Encapsulation in Silk Nanoparticles

Thioflavin T was used as the model drug for loading studies. To prepare Thioflavin T-loaded silk nanoparticles, aqueous silk mixture was supplemented with 14.3 μM Thioflavin T, following silk nanoparticle manufacture. The resulting SNPs were collected and analyzed using NTA in the light scattering mode, followed by acquisition in the fluorescent mode with a minor modification for the focus setting. The proportion of empty particles was determined by subtracting the size distribution profile measured in fluorescence mode—which detects Thioflavin T-loaded particles—from that measured in light scattering mode, which detects all particles. The Thioflavin T loading efficiency was calculated as follows:

$$\text{Loading efficiency (\%)} = \frac{\text{Particle concentration of silk nanoparticles (fluorescence mode)}}{\text{Particle concentration of silk nanoparticles (scattering mode)}} \times 100 \quad \text{Equation 3.4}$$

3.2.12. Cytotoxicity of Silk Nanoparticles

The cytotoxicity of silk nanoparticles was investigated in RAW 264.7 murine macrophages (ATCC, product number TIB-71TM, ATCC, UK). Briefly, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v FBS, 50 U/mL penicillin, and 50 mg/mL streptomycin. When the cells reached 80–90% cell confluency, they were scraped from the plates, centrifuged at 380g for 4 min, seeded into a 96-well plate at a cell density of 15,000 cells/cm², and incubated in a 5% v/v CO₂ incubator at 37 °C for 24 h. The culture medium was then replaced with fresh medium containing 31–500 µg/mL of silk nanoparticles, and the cells were cultured for 48 h. Cell viability was determined by adding 20 µL of 5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide solution, incubating for 4 h, and reading the absorbance at 570 nm (Multiskan Ascent V1.24, Thermo Fisher Scientific Inc., MA, USA). Cells treated with blank complete medium served as untreated control cells. Percentage viability was expressed relative to the untreated control, and a cytotoxicity assay was performed with three biological replicates ($n = 3$).

3.2.13. Inflammation Induction by Silk Nanoparticles

RAW 264.7 murine macrophages were processed as described earlier. After plating overnight, cells were exposed to fresh medium containing 500 µg/mL silk nanoparticles. Cells treated with 200 ng/mL lipopolysaccharide (LPS) served as positive inflammatory-activated control cells. After a 24 h incubation, medium was collected and centrifuged at 21,630g for 5 min (VWR® Micro Star 30 centrifuge, VWR International, LLC., Leicestershire, UK). The resulting supernatant was transferred to a low-binding protein microtube and preserved at –80 °C for further analyses.

Endogenous nitrite (NO_2^-) was measured using a Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit (R&D Systems, Minneapolis, MN, USA). Tumor necrosis factor alpha ($\text{TNF-}\alpha$) was measured using a Mouse TNF-alpha Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). Cytokine and chemokine profiles were semi quantitatively measured using Proteome ProfilerTM Array (Mouse Cytokine Array Panel A, R&D Systems, Minneapolis, MN, USA). All cell-based datasets were conducted as three independent cell experiments ($n = 3$).

3.2.14. Analysis Software and Statistical Analyses

Data were compiled using Microsoft Excel version 16.92 (Microsoft Office 365 for Mac, Redmond, WA, USA). FTIR data were processed using Origin 2019b (OriginLab, Northampton, MA, USA), and the NMR spectra were analyzed and plotted using MestReNova (Mestrelab Research, Santiago de Compostela, Spain). Image J (NIH National Institute of Mental Health, Bethesda, MD, USA) was used to derive size and circularity parameters from scanning electron microscopy micrographs and for cytokine dot intensities on cytokine membranes. Graphs and statistical analyses were prepared using GraphPad Prism 10 (GraphPad Software, Boston, MA, USA). Statistical tests included one-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks indicate statistical significance, and the number of experimental repeats (n) is provided in the figure legends.

3.3. Results

3.3.1. Silk Structural and Conformational Changes

Thioflavin T fluorescence and NMR were used to investigate the impact of K^+ inclusion on liquid silk structural and conformational changes. Thioflavin T fluorescence intensity increased in the positive control of IPA-treated silk fibroin, while no significant changes were observed

in K⁺ incorporation for both 1.1 mg and 17.3 mg K⁺ (**Figure 3.2a**). NMR data revealed no significant chemical shifts in the NH region (8.5–7.8 ppm) of 1D ¹H-NMR spectra; however, slight changes in the spectra were present in 1.1 mg and 17.3 mg K⁺ compared to control, showing overall increases of 4.1% and 5.2%, respectively (**Figure 3.2b**). The 2D [¹H, ¹⁵N] HSQC NMR spectra displayed ¹⁵N chemical shifts for amino acids in a random coil state, corresponding to glycine (108.8 ppm), serine (115.7 ppm), alanine (123.8 ppm), and an unidentified amino acid (110.8 ppm) (**Figure 3.2c**).¹⁹⁸

FTIR analysis was performed to track changes in the secondary structure of silk from liquid silk to silk nanoparticles prepared using K⁺ at low and high mass ratios (1.1 and 17.3 mg). In the absence of K⁺, the silk nanoparticles exhibited a higher degree of β -sheet structure than the K⁺ silks; however, all silk nanoparticles displayed a typical shift in the IR absorbance spectrum at ~1640 to ~1620 cm⁻¹ (the amide I region) associated with the β -sheet structure (**Figure 3.2d**).²⁵¹ This result was consistent with an increase in the total β -sheet content, from 20.21% to 61.16%, for silk nanoparticles (**Figure 3.2e**). This suggests that my nanoparticles exhibited a β -sheet-rich structure that was nearly identical to a silk II reference (a silk film treated with 10% ethanol), consisting of 61% β -sheet, 26% β -turn, and 13% random coil. Interestingly, the incorporation of 1.1 and 17.3 mg K⁺ in silk nanoparticles and liquid silk had no significant impact on the secondary structure content, as the nanoparticles and liquid silk structures were maintained close to those of their silk II and silk I references, respectively (**Figure 3.2e** and **Figure 3.3**). The correlation coefficient (R) for the nanoparticles, obtained from the second derivative amide I spectra and indicating formulation-induced structural changes in the silk nanoparticles relative to air-dried silk films (silk I reference), ranged between 0.308 and 0.376, similar to the silk II reference value of 0.232 (**Figure 3.2f**).

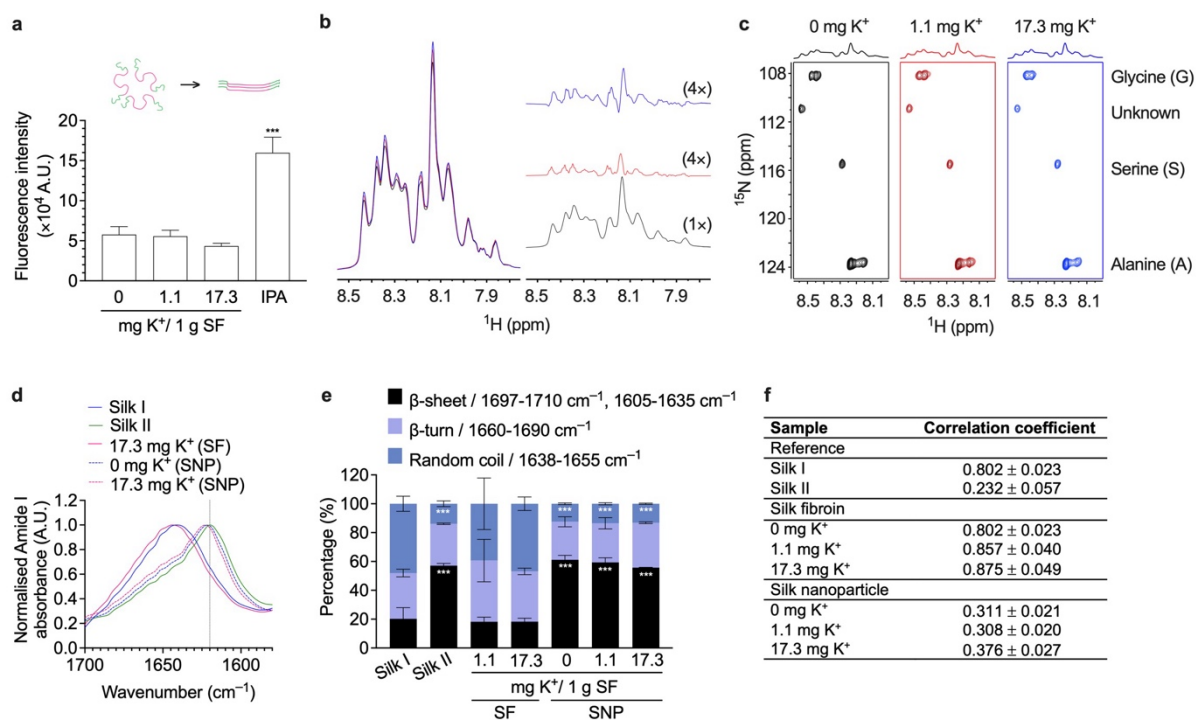


Figure 3.2 Analysis of the structure and conformation of aqueous silk and solid nanoparticles. (a) The effect of K⁺ on aqueous silk fibroin (SF) structural alteration measured by Thioflavin T (ThT) fluorescence ($n = 3$), (b) 1D ¹H-NMR ($n = 1$), (c) and 2D [¹H, ¹⁵N] HSQC NMR spectra in the NH region ($n = 1$). The 1D ¹H-NMR spectra (left panel), with the difference spectra (red and blue, right panel) displayed for comparison to the control spectrum (black). Black traces represent the control, red corresponds to 1.1 mg K⁺ (4.1% increase), and blue to 17.3 mg K⁺ (5.2% increase). (d) FTIR secondary structures of freeze-dried aqueous silk (17.3 mg K⁺) and silk nanoparticles (SNP) (0 and 17.3 mg K⁺), (e) secondary structure content, (f) and correlation coefficient ($n = 3$). The second-derivative amide I spectrum of an air-dried silk film (silk I) served as a reference for the correlation coefficient (R) calculations (mean $\pm$ SD, $n = 3$). One-way ANOVA and Dunnett's multiple comparisons test for the Thioflavin T assay; Two-way ANOVA and Dunnett's multiple comparisons test for secondary structure content; $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (**). All experiments were conducted as three independent experimental repeats, and three technical repeats for FTIR study ($n = 3$).

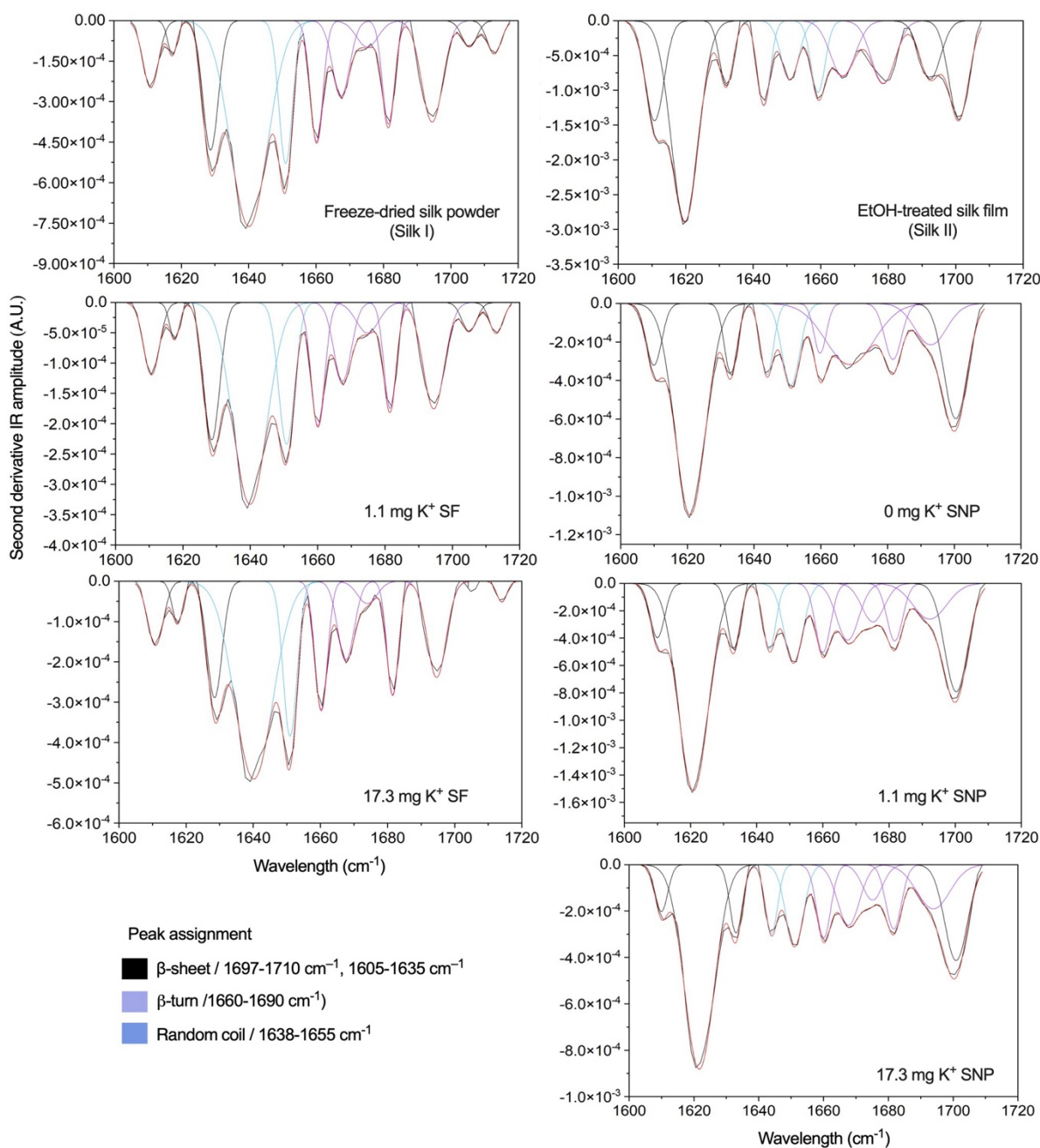


Figure 3.3 Exemplar IR fitting of the amide I region performed for K^+ -mixed silk fibroin (1.1 and 17.3 mg K^+ SF) and K^+ -mixed silk nanoparticles (SNP) (0, 1.1, and 17.3 mg K^+ SNP), in comparison with freeze-dried silk powder (silk I) and EtOH-treated silk film (silk II) controls. The freeze-dried silk powder (silk I) serves as the 0 mg K^+ -mixed silk. The experiment was conducted as three independent experimental repeats ($n = 3$).

3.3.2. Silk Nanoparticle Physicochemical Properties

Silk nanoparticles were fabricated using low and high K⁺ mass ratios (1.1 and 17.3 mg), and particle size and polydispersity index (PDI) were assessed using DLS and NTA. Particle size measurements from DLS were consistent with those from NTA (in brackets), showing a slight increase in particle size with higher K⁺ mass ratios compared to 0 mg K⁺ control silk nanoparticles, from 92.98 d.nm (78.81 d.nm) to 105.3 d.nm (80.71 d.nm) for 1.1 mg K⁺, and 290.6 d.nm (171.9 d.nm) for 17.3 mg K⁺. All silk nanoparticles had a favorable PDI of <0.15.^{200, 224} However, a significant difference in size between DLS and NTA data was observed for the 17.3 mg K⁺ nanoparticles, which showed a reduction in particle size of ~1.7-fold when determined using NTA, while the control particles and 1.1 mg K⁺ particles exhibited size reductions of ~1.2-fold and 1.3-fold, respectively. Despite the larger particle size, the 17.3 mg K⁺ silk nanoparticles had a lower particle concentration compared to the other ones (**Figure 3.4a**).

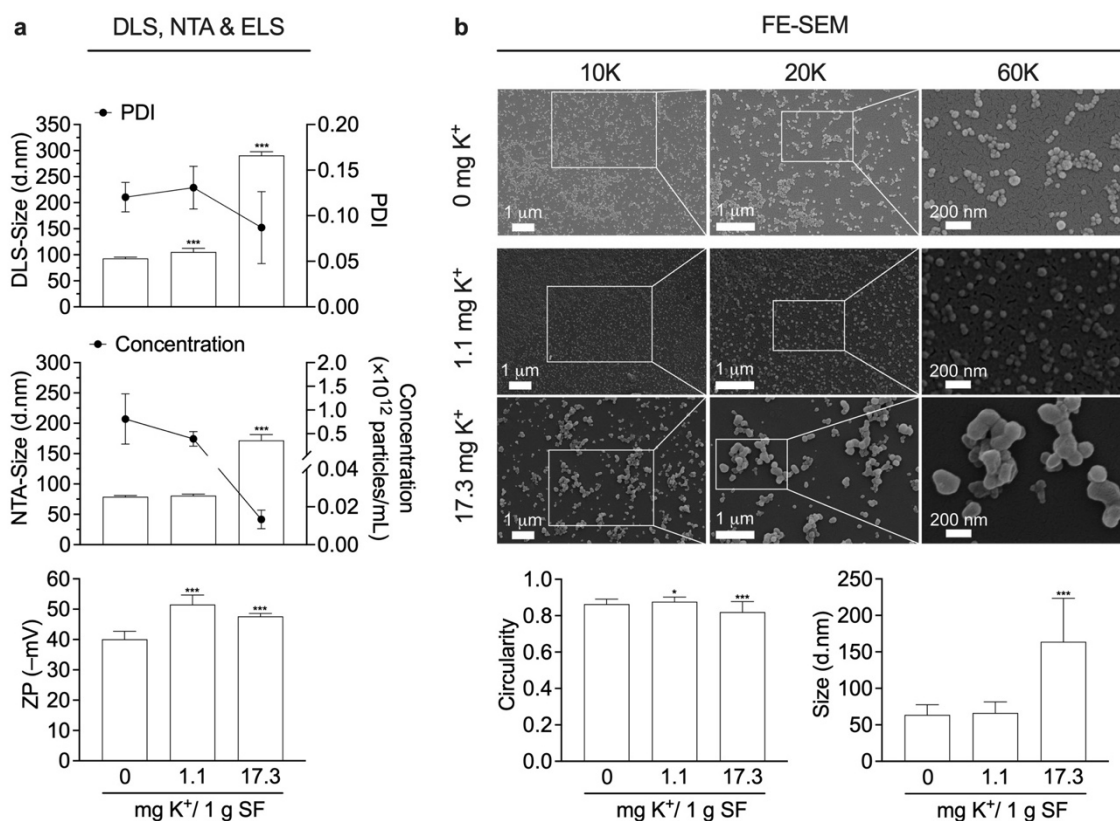


Figure 3.4 Silk nanoparticle physicochemical properties. (a) Analysis of particle size by DLS and NTA and zeta potential (ZP) by ELS ($n = 3$). (b) FE-SEM images taken at 10, 20, and 60K magnifications and corresponding size and circularity for 400–500 particles and 100–150 particles, respectively. One-way ANOVA and Dunnett's multiple comparison test were used for statistical analysis: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). DLS, NTA, and ZP were conducted as three independent formulation batches and three technical repeats each ($n = 3$). FE-SEM was conducted as one experimental repeat with three technical repeats for computed circularity and size analyses ($n = 1$).

FE-SEM micrographs showed that increasing the K⁺ mass ratio led to larger particle sizes (63.56–163.70 d.nm), while particle morphology remained largely unaffected, with circularity values consistently approximating 1 (0.82–0.87), indicating near-spherical shapes (**Figure 3.4b**). In addition to the particle size, the particle net charge surface was significantly altered by K⁺, as indicated by the increase in zeta potential (ZP) from –40 mV to –52 mV for the 1.1 mg K⁺ SNPs and to –48 mV for the 17.3 mg K⁺ particles (**Figure 3.4a**).

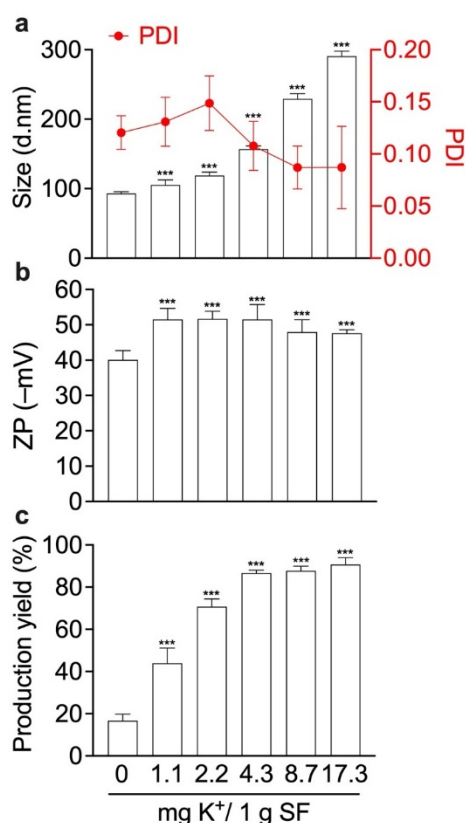


Figure 3.5 Silk nanoparticle physicochemical properties. (a) Analysis of particle size using DLS (b) zeta potential (ZP) by ELS, and (c) production yield calculated by SNP dried weight ($n = 3$). Silk nanoparticle was manufactured in the K^+ mass range of 1.1 to 17.3 mg. The One-way ANOVA and Dunnett's multiple comparison test were used for statistical analysis: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). All experiments were conducted as three independent formulation batches and three technical repeats each ($n = 3$).

The two-fold increase in the K^+ mass ratio—from the lowest at 1.1 mg to the highest at 17.3 mg—was also investigated, revealing a ratio-dependent effect of K^+ on size tuning and increased production yield, while maintaining PDI value (<0.15) and negligible reduction of zeta potential (Figure 3.5).

3.3.3. Model Drug-Loaded Silk Nanoparticle

Thioflavin T-loaded nanoparticles were detected using NTA, revealing an increase in the Thioflavin T-loading percentage from 76.24% for the control silk nanoparticles (0 mg K^+) to 78.27% for the 1.1 K^+ silk nanoparticles and to 80.10% for the 17.3 mg K^+ SNPs (Figure 3.6a). For the same protein concentration (1 mg/mL), increasing K^+ mass ratios increased the mean

particle size from 100.27 (± 1.36) for the control particles to 95.67 (± 2.89) for the 1.1 K⁺ particles and to 190 (± 2.11) d.nm for the 17.3 mg K particles. In contrast, fewer particles were detected after 60 s, consistent with the increased K⁺ mass ratios from 7.96×10^{13} particles for the control to 13.5×10^{13} for the 1.1 K⁺ nanoparticles and 1.9×10^{13} particles for the 17.3 K⁺ nanoparticles (**Figure 3.6b**).

The fluorescence intensity detected from Thioflavin T-loaded silk nanoparticles indicated that Thioflavin T was more efficiently entrapped in larger particles fabricated with a high K⁺ mass ratio (7.4 A.U.), whereas the median intensity showed no significant changes with the low K⁺ mass ratio silk nanoparticles (4.1 A.U.) compared to the 0 K⁺ nanoparticles (4.0 A.U.) (**Figure 3.6c**).

Comparison of the production yield of Thioflavin T-loaded particles to particles fabricated without Thioflavin T also revealed that addition of Thioflavin T reduced silk nanoparticle production yield by factors of 1.35, 1.86, and 1.14 for the control, 1.1 mg K⁺, and 17.3 mg K⁺ particles, respectively. However, the manufacturing conditions used can significantly reduce silk biomaterial waste, potentially increasing the yield by 2.8-fold (1.1 mg K⁺ silk nanoparticles) and 5.8-fold (17.3 mg K⁺ silk nanoparticles) compared to control silk nanoparticles during empty particle production (without payloads). Similarly, the yield for Thioflavin T-loaded particle production was 2.1-fold and 6.9-fold greater than for control silk nanoparticles (**Figure 3.6d**).

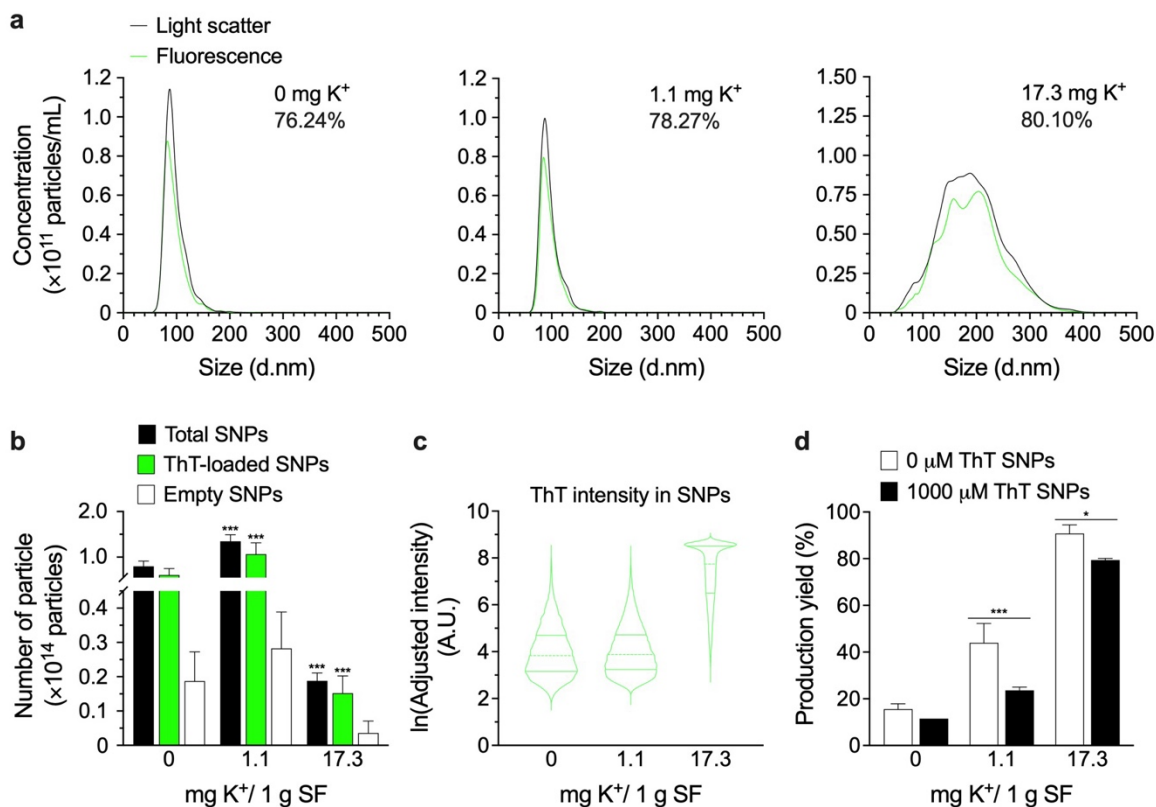


Figure 3.6 Model drug-loaded silk nanoparticles (SNPs) using Thioflavin T as a drug model. Thioflavin T-loaded SNPs were prepared and characterized by NTA (a) size distribution profile measured by light scattering mode (black) and fluorescence mode (green); (b) the particle number; (c) Thioflavin T fluorescence intensity. (d) The production yield was calculated according to the SNP dried weight. One-way ANOVA and Dunnett's multiple comparison tests were used for statistical analysis: $p < 0.05$ (*), $p < 0.01$ (), and $p < 0.001$ (***) . The NTA experiment was conducted as three independent experimental repeats ($n = 3$).**

3.3.4. Cytotoxicity and Inflammation of Silk Nanoparticles

The cytotoxicity of silk nanoparticles was investigated in macrophages within the 31–500 $\mu\text{g/mL}$ concentration range. All silk nanoparticles showed similar cytotoxicities, as indicated by reductions in cell viability from 99% to 75% in parallel with increased silk nanoparticles concentrations (**Figure 3.7a**). Silk nanoparticles at the highest nanoparticle dose (500 $\mu\text{g/mL}$) triggered negligible release of cytokines and chemokines from macrophages and the response was similar to baseline. In contrast, cells treated with 200 ng/mL LPS (positive control inflammation-stimulated cells) exhibited excessive release of inflammation-related cytokines and chemokines. Notably, changes in expression levels due to silk nanoparticle treatment were observed only for RANTES, MIP-2, TNF- α , SDF-1, MIP-1 β , MIP-2, JE, IP-10, IL-1ra, IFN- γ , and ICAM-1. The expression of other inflammatory markers (e.g., TREM-1, TIMP-1, IL-27, IL-10, IL-6, IL-1 α , GM-CSF, and G-CSF) was not elevated in silk-treated cells (**Figure 3.6b** and **Figure 3.8**). Overall, exposure to silk nanoparticles resulted in negligible cytokine and chemokine release, with expression levels remaining near baseline. No significant increases in NO₂⁻ (1.2 vs. 1.3 μM) or TNF- α (0.02 vs. 0.06 ng/mL) were observed when comparing cells before vs. after treatment with silk nanoparticles at the highest dose of 500 $\mu\text{g/mL}$. Conversely, treatment with 200 ng/mL LPS promoted an excessive level of both NO₂⁻ (4.9 μM) and TNF- α (5.02 ng/mL) (**Figure 3.6c**).

In addition, to evaluate and compare the impact of K⁺ and Ca²⁺ on the modulation of liquid silk structure and conformation, as well as on the physicochemical attributes of silk nanoparticles, the correlation coefficient (r) between the measured variables in the K⁺ dataset (this chapter) and the reference Ca²⁺ dataset (chapter 1²⁴⁵) ($n = 3$) was calculated (**Figure 3.9**).

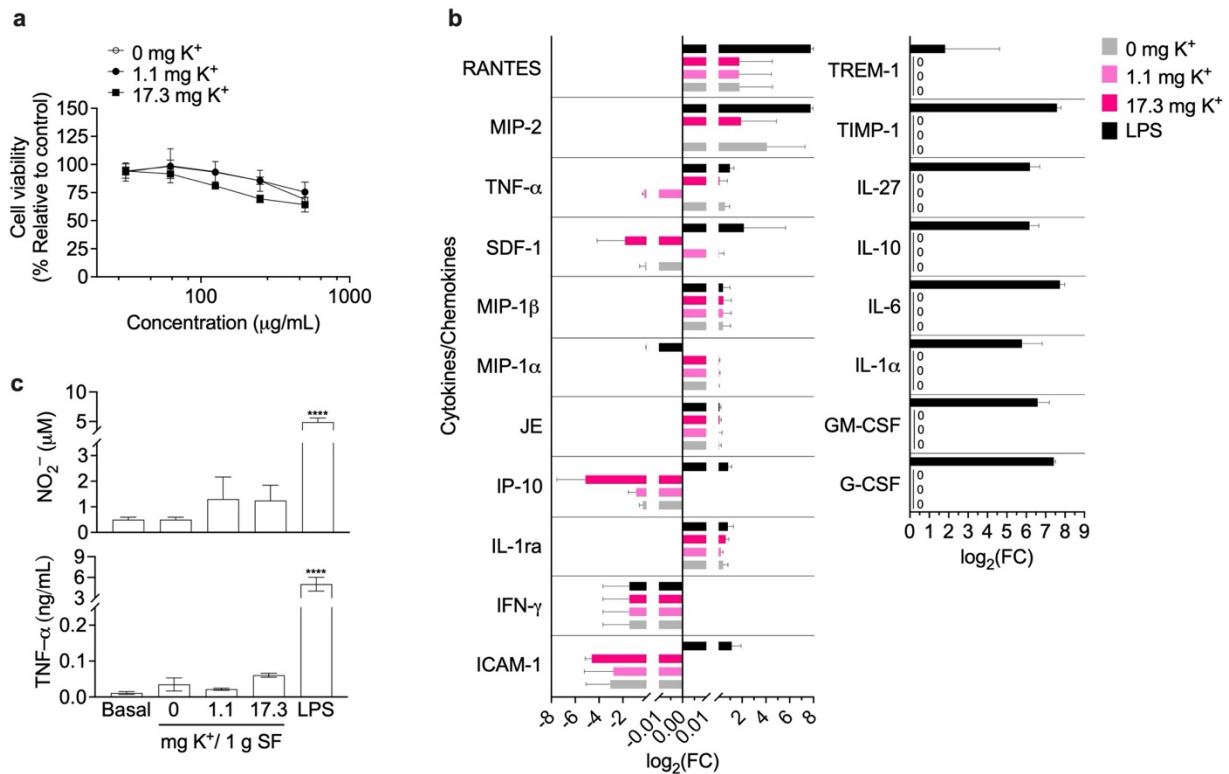


Figure 3.7 Cellular responses of RAW 264.7 murine macrophages to silk nanoparticles (SNPs). (a) Cytotoxicity of macrophages exposed to SNP levels ranging from 31.25 to 500 $\mu\text{g/mL}$ for 48 h ($n = 3$). Inflammatory responses: (b) release profile of cytokines and chemokines presented in $\log_2$ fold-change (FC) of expression level compared with a basal control. Interpretation of $\log_2(\text{FC})$: a value of zero indicates no change in expression, positive values indicate upregulation, and negative values indicate downregulation ($n = 3$); and (c) NO_2^- and $\text{TNF-}\alpha$ production in macrophages exposed to 500 $\mu\text{g/mL}$ SNPs for 24 h ($n = 3$). Cells treated with 200 ng/mL LPS served as the positive inflammation-stimulated control. Cells exposed only to the complete medium served as negative control. One-way ANOVA and Dunnett's multiple comparison test were used for statistical analysis: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). All cell-based assay was conducted as three independent cell experiment repeats ($n = 3$).

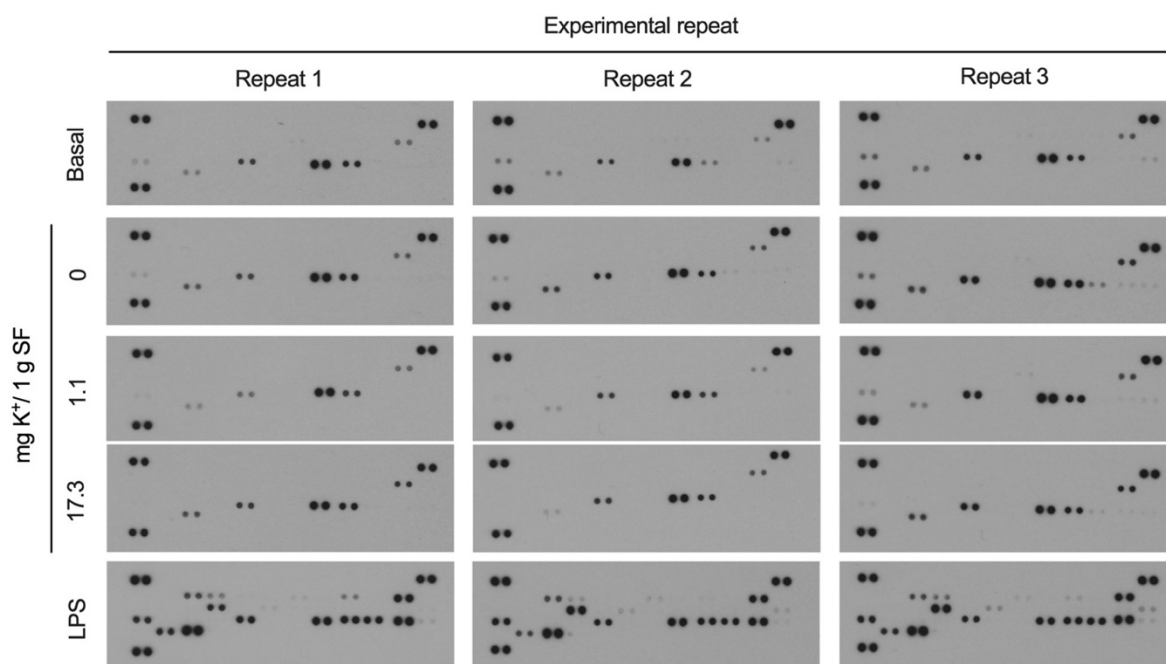


Figure 3.8 Inflammatory cytokine levels were measured in RAW 264.7 murine macrophages in response to silk nanoparticle exposure. Cytokine expression data were collected from three independent experimental repeats. Macrophages were treated with either complete media or media containing 200 ng/mL lipopolysaccharide, which served as the basal and positive controls, respectively. This experiment was carried out alongside the calcium silk nanoparticle,²⁴⁵ with the same set of controls used for both. The experiment was conducted as three independent cell experiment repeats ($n = 3$).

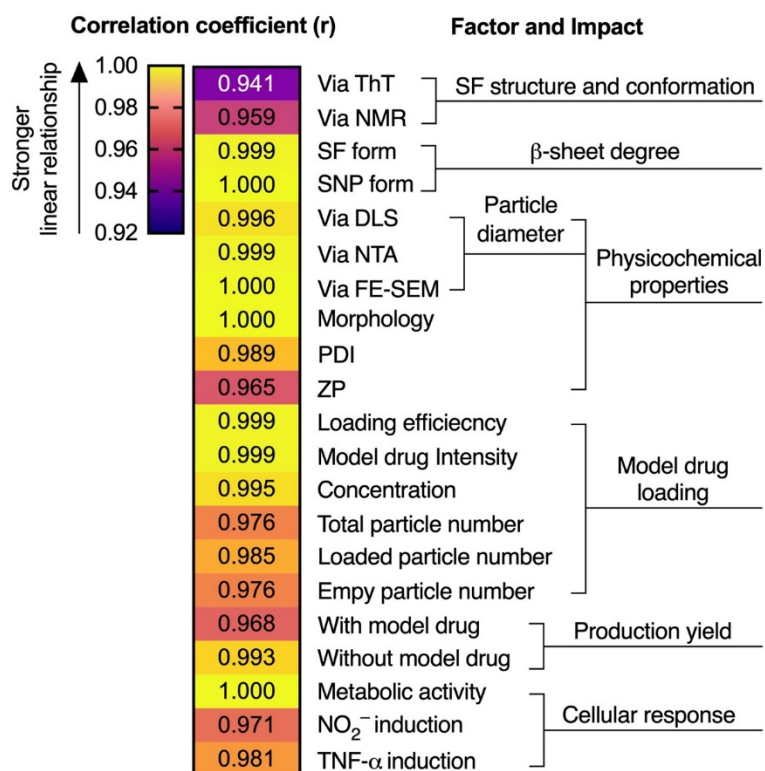


Figure 3.9 Impact of calcium and potassium on silk nanoparticle performance. The correlation coefficient (r) between the measured variables in the K⁺ dataset (this study) and the reference Ca²⁺ dataset (previous study²⁴⁵) ($n = 3$): positive linear correlation ($r > 0$), non-linear correlation ($r = 0$), and negative linear correlation ($r < 0$).

3.4. Discussion

3.4.1. The Impact of K^+ on Silk Structural and Conformational Changes

This study explored the role of K^+ in silk nanoparticle assembly deploying physicochemical solution and solid-state techniques. The Thioflavin T assay was used to assess the impact of K^+ on liquid silk structural and conformational changes. Thioflavin T is a fluorescent dye widely used for the determination of protein aggregation/hydrophobicity, structural and conformational alterations, and protein interactions.²⁵² The intercalation of Thioflavin T with the β -sheet of the amyloids creates a strong fluorescence signal at 480 nm.²⁵³ The observed increase in fluorescence intensity in the isopropanol-treated aqueous SF condition indicated the bound Thioflavin T state and served as a positive control for silk β -sheet structure and/or the induction of silk aggregation. However, the use of 1.1 mg or 17.3 mg K^+ did not significantly alter the silk structure or aggregation compared to the control (0 mg K^+), suggesting that Thioflavin T assay was not able to uncover subtle differences in liquid silk interactions and structural property determinations (**Figure 3.2a**). The impact of calcium ions on the liquid silk structure, which was also studied with the Thioflavin T assay is discussed in Chapter 1.²⁴⁵

I also used NMR to explore silk behavior in solution but observed no significant chemical shifts in the NH region (8.5–7.8 ppm) of 1D 1H -NMR spectra. However, a similar trend to Ca^{2+} was observed, as the K^+ mass ratio increased, resulting in an increased peak intensity and altered peak sharpness. Compared to the control (0.0 mg K^+), the spectra for silk formed in the presence of 1.1 mg and 17.3 mg K^+ displayed overall increases of 4.1% and 5.2%, respectively (**Figure 3.2b**). I hypothesize that this increased signal reflected alterations in the exchange rate of labile NH protons. This increase in the NMR signal suggests that a larger proportion of the labile hydrogens are located in the same local NH chemical environment, implying a change in protein stability.²¹⁸

The amino acids revealed by the 2D [^1H , ^{15}N] HSQC NMR spectra included glycine, alanine, and serine as the major components of *B. mori* silk (**Figure 3.2c**),¹⁹⁸ as reported elsewhere,²⁵⁴ and accounted for 45.6%, 29.8%, and 10.7% by mol, respectively. Thus, a notably high intensity was noted for the 2D [^1H , ^{15}N] HSQC NMR spectra contour plot. The hydrophobic amino acids, consisting of glycine-X repeats,^{2, 255} play an important part in the anti-parallel β -sheet structure of silk. However, the regulation relevant to the anti-parallel β -sheet structure could be identified according to the main roles of these amino acids: glycine maintains proper packing/assembly,¹¹ alanine maintains stability and rigidity,²⁵⁶ and serine provides hydrophobicity.²⁵⁶ Taken together, the findings suggest that an increase in the proportion of labile hydrogens may predominantly occur in the NH microenvironment of these major amino acids to affect the formation as well as the rigidity of the β -sheet structures, leading to increased aggregation/assembly of the silk fragments.

The FTIR findings suggest that K^+ mass ratios ranging from 1.1 to 17.3 mg (equivalent to 1 to 17 mM KCl) may not be sufficient to directly influence the structural rearrangement of liquid silk. This was consistent with a previous report showing that higher KCl concentrations (> 0.1 M KCl) increased the β -sheet content in spider silk.²⁵⁷ One possible explanation is that silk-ion binding interactions in this system may be moderated and saturated in liquid silk, which reaches the maximum β -sheet structure for silk nanoparticles (55.71–61.16%). However, the NMR data prompted further investigation of the effects of K^+ on silk conformation and assembly, particularly with respect to the modulation of nucleation, saturation, and particle growth during anti-solvent precipitation.

3.4.2. Impact of K^+ on Silk Nanoparticle Physicochemical Properties

These findings demonstrate that altering K^+ mass ratios enable the tuning of silk nanoparticle size, with the hydrodynamic diameter increasing as the K^+ mass ratio rises. This result may be attributed to the influence of K^+ on the physicochemical properties of silk, suggesting that an

optimal K^+ mass of < 4.3 mg is favorable for achieving particle diameters <200 nm, prolonging circulation half-life: eliminated by kidneys (<10 nm) and promoted clearance pathway (>200 nm).^{258, 259} Although larger in size, 200–300 nm particles may be useful for nanoparticle-mediated topical drug delivery.²⁶⁰ NTA measures particle size on a particle-by-particle basis,¹¹⁷ while DLS evaluates the sample collectively as an ensemble.¹¹² Compared with DLS, NTA provides higher resolution for polydisperse systems and enables direct visualization of particles, allowing aggregation behavior to be more easily identified.¹¹⁸ This study showed smaller particle sizes by NTA, suggesting that silk nanoparticles underwent greater aggregation under the DLS measurement conditions. (**Figure 3.4a**). This observation was consistent with the FE-SEM micrographs, which confirmed an increase in the particle size with an increase in the K^+ mass ratio from 63.56 d.nm to 66.17 d.nm for 1.1 mg K^+ silk nanoparticles and 163.70 d.nm for 17.3 mg K^+ silk nanoparticles. Interestingly, K^+ had a negligible effect on particle morphology, as the circularity value was close to 1 (0.82–0.87), similar to that of a perfect sphere (**Figure 3.4b**).²⁶¹

One possible explanation for the observed effects of K^+ involves nucleation, where small protein clusters initially aggregate.^{23, 220} Increasing K^+ concentration shields the negative charge of silk fragments, reduces electrostatic repulsion, disrupts hydration layers, and alters ionic strength, thereby promoting protein folding and silk self-assembly.^{15, 110, 246, 247, 262} During particle growth, K^+ may further enhance aggregate formation, leading to larger clusters in the presence of isopropanol. These findings suggest that nanoparticle size is regulated by K^+ in a concentration-dependent manner.

3.4.3. Impact of K^+ on Model Drug Encapsulation

The interaction between the silk carrier and Thioflavin T as a model compound was explored in this chapter, with simultaneous assessment of beta sheet structure and payload loading efficiency influenced by K^+ . This enabled the detection of beta-sheet structures and provided

fluorescence for easy detection using nanoparticle tracking analysis in the fluorescence and light scattering modes. The size distribution data from fluorescence and light scattering modes allows for the calculation of Thioflavin T entrapment efficiency by comparing particle concentrations.²⁶³

K⁺ inclusion promoted the intercalation of Thioflavin T within the silk assembly/aggregates, as Thioflavin T preferentially interacts with silk hydrophobic regions (e.g., aggregates and β -sheet structures),²⁵² leading to increased Thioflavin T entrapment in larger-sized particles.

3.4.4. Cellular Responses to K⁺-Silk Nanoparticle

This thesis showed the high biocompatibility of silk nanoparticles in a macrophage cell line, with no significant induction of inflammatory responses observed following exposure to silk nanoparticles. I hypothesised that the electrostatic interaction network formed by the counterion^{15, 110, 246, 247} contributes to stabilization of the β -sheet-rich structure. In combination with a negative surface charge, this improves particle integrity and colloidal stability, preserves size and shape, reduces aggregation, and lowers cytotoxicity, as unstable particles may increase cellular risk.²⁶⁴ Furthermore, particle size and surface charge may influence cellular responses. With similar zeta potentials across samples, smaller particles exhibit higher charge density, which can enhance particle–cell interactions, potentially disrupting membrane integrity and inducing programmed cell death pathways.²⁶⁵ However, a slight reduction in cell viability was observed with largest (17.3 mg K⁺) silk nanoparticles, suggesting that charge density was of limited biological importance. Overall, these findings demonstrate that the inclusion of K⁺ generated silk nanoparticles that exhibited high biocompatibility with macrophages, negligible cytotoxicity, and minimal induction of inflammation. However, limitations of using RAW 264.7 macrophages should be considered, as their phagocytic and inflammatory responses may differ from primary or human macrophages, potentially affecting nanoparticle uptake,

inflammation silencing, and toxicity results. Endotoxin contamination should also be assessed, as it may influence macrophage responses and limit biomedical translation.

3.4.5. Impact of Ions on the Modulation of Silk Nanoparticle Properties

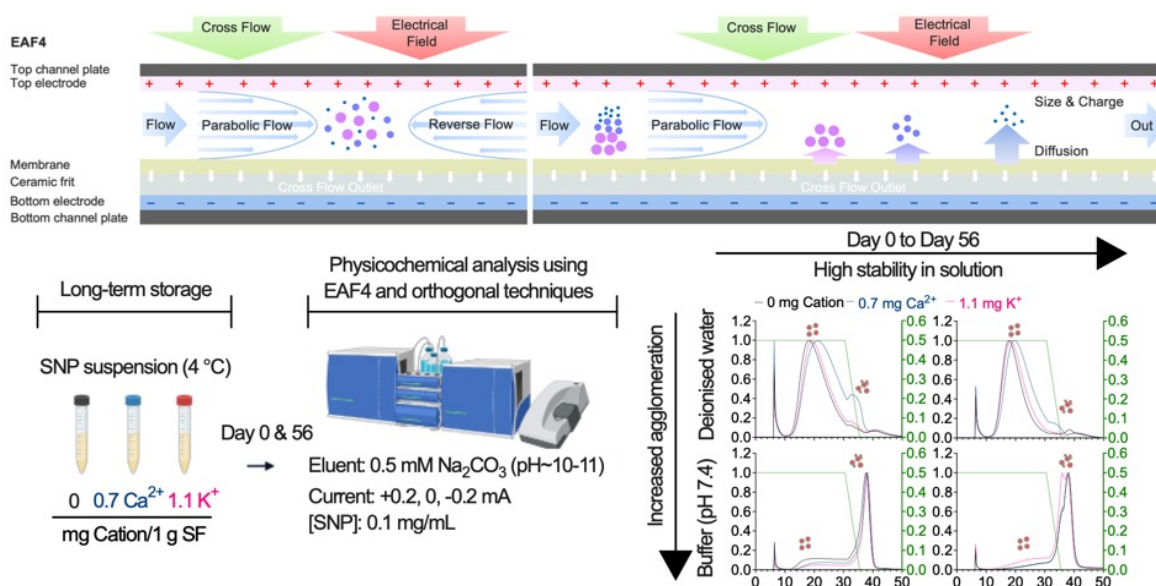
The combined effects of several metal elements and their physiological concentrations can interact synergistically or antagonistically to influence silk fibroin behavior in the natural spinning environment by regulating its structural transition, stability, and assembly.²⁸ In a previous study, I investigated the effect of Ca^{2+} on silk nanoparticles within the same manufacturing system and established a fine-tuning process model with optimized physicochemical properties. In the present study, I focused on the role of K^+ , which is among the most abundant of metal elements. Unlike Ca^{2+} , K^+ differs in both valency and its concentration gradient along the silk gland. While Ca^{2+} decreases from the posterior to the anterior region, K^+ shows a significant increase in the anterior part.²⁸ I analyzed the correlation for each key variable factor between K^+ particles and reference Ca^{2+} particles using correlation coefficients (**Figure 3.9**). This result demonstrated that K^+ and Ca^{2+} both modulated particle size, shape, and loading efficiency in a consistent and correlated manner, suggesting complementary mechanisms. Nonetheless, Ca^{2+} had a greater impact on modulating silk fibroin behavior, promoting more assembly and aggregation and improving production yield by reducing silk fibroin wastage.²⁴⁵ I hypothesized that in the aqueous silk state, Ca^{2+} has a greater impact than K^+ in altering aqueous silk fibroin behavior, as evidenced by Thioflavin T assay and NMR analysis, which showed a difference of up to 17.5% for Ca^{2+} compared to 7% for K^+ . This could be attributed to differences in valency and ionic radius, with Ca^{2+} being more effective than K^+ at forming strong electrostatic interactions with proteins.^{15, 36, 110, 248, 266} However, K^+ had a much lower impact on disrupting silk nanoparticle surface charge, whereas Ca^{2+} reduced the ZP by approximately half. Together, these findings show that mimicking the natural roles of K^+ (chapter 3) and Ca^{2+} (chapter 2)²⁴⁵ improves the physicochemical properties

and scalability of silk nanoparticles. These distinct effects highlight the potential of exploring synergistic or antagonistic interactions among multiple metal ions in silk nanoparticle formulation to achieve more tailored properties.

3.5. Conclusion

Silk nanoparticles were manufactured using the anti-solvent precipitation method in a semi-batch format to orchestrate nanoparticle assembly. These findings demonstrated that K^+ can modulate the physicochemical properties of silk, both in liquid form and as solid silk nanoparticles. Specifically, K^+ induced changes in silk self-assembly, promoted the formation of β -sheet-rich cores, enabled tunable particle size, enhanced payload encapsulation efficiency, and favourable shifts in particle net surface charge (zeta potential). The addition of K^+ also improved formulation efficiency by increasing yield and reducing silk loss during production. The resulting β -sheet-rich silk nanoparticles exhibited low toxicity and minimal inflammatory activity, supporting their potential as biocompatible drug nanocarriers. Collectively, these findings offer a practical strategy for fine-tuning silk-based nanomaterials for pharmaceutical applications. Future work should assess loading capacity for diverse drug types, optimize semi-batch scale-up, and evaluate nanoparticle stability under varied storage conditions to support clinical and commercial translation.

Chapter 4 Evaluating the impact of bioinspired counterion inclusion on silk nanoparticle physicochemical attributes and physical stability



Chapter 4 presents the results that were published in *Nanoscale Advances* (Roamcharern*, N.; Punnabhum, P.; Seib, F. P.; Rattray, Z. Evaluating the impact of bioinspired counterion inclusion on silk nanoparticle physicochemical attributes and physical stability, *Nanoscale Advances*, 2025, 7(18), 5519-5535. (DOI: <https://doi.org/10.1039/D5NA00365B>). This work focused on the physicochemical stability test of silk nanoparticles, highlighting their colloidal stability in solution under influencing factors of ionic strength and pH. The short-term and long-term storage stability were investigated using various orthogonal techniques, including DLS, NTA, ELS, FE-SEM, and EAF4. I contributed to the preparation of silk nanoparticles, conducted all experiments, and was responsible for data analysis and graphing. Panida Punnabhum provided training on the NTA and EAF4 instruments. In addition to hardware and software training, Punnabhum also assisted in method development for the EAF4 study. Dr. Zahra Rattray and Dr. Philipp Seib were responsible for the study conceptualization and design, data analysis, manuscript revisions, and securing funding.

4.1. Introduction

Nano-sized drug delivery systems have gained substantial attention as vehicles for improving targeted bioactive drug delivery and the controlled release of biomedical and pharmaceutical therapies.² Bioinspired protein-based nanocarriers offer distinct advantages for nanoscale drug delivery applications.²⁶⁷ For example, silk fibroin from *Bombyx mori* stands out due to its favorable physicochemical properties, including mechanical strength,²⁶⁸ biocompatibility,²⁴ biodegradability,²⁴ and broad-spectrum applications in nanostructured biomaterials processing for nanomedicine.²⁶⁹

The self-assembly of silk fibroin and the thermodynamic processes that regulate it have been a focus of past research. Emerging evidence confirms that this assembly is influenced by the molecular mobility, charge, hydrophilic interactions, and concentration of silk fibroin.²⁷⁰ Thus, modifying the physicochemical properties of silk fibroin can create new nanostructure architectures and conformations, thereby enhancing the manufacturability of silk nanoparticles.

Silk nanoparticles can be generated using various bottom-up protocols, including desolvation, coacervation, emulsification, antisolvent precipitation, nano spray drying, and electrospraying.²⁶⁷ Antisolvent precipitation involves the rapid desolvation of soluble silk proteins in a solvent to form protein precipitates that can entrap drug cargoes.²⁷¹ The physicochemical attributes of the resulting nanoparticles are influenced by the choice of protocol parameters, including organic solvent composition, volume ratio of non-solvent to organic solvent, injection speed, and stirring rate.^{271, 272} In a previous study, I successfully manufactured silk nanoparticles using a antisolvent precipitation method in a semi-batch format that included incorporation of Ca^{2+} and K^{+} cations to improve silk nanoparticle production yield. This method resulted in a range of critical nanoparticle quality attributes, with a particle diameter in the 93–290 nm range, a polydispersity index <0.15 , and zeta potential in

the -39 to -52 mV range.²⁴⁵ These properties of silk nanoparticles, together with their negligible cytotoxicity and undetectable induction of inflammatory responses in macrophages, suggested that silk nanoparticles are promising for biomedical and pharmaceutical applications. My further FTIR and NMR studies of the impact of Ca^{2+} on silk fibroin solution-phase properties demonstrated structural transitions from liquid silk (silk I) to solid silk nanoparticles (silk II).²⁴⁵

Despite their potential, nanoparticles for therapeutic applications face numerous translational challenges related to their physical properties, such as colloid stability, swelling behavior, and crystallinity.²⁷³ Loss of physical stability, particularly colloidal stability, negatively impacts the biological fate of protein-based nanocarriers *in vivo* by triggering immunogenicity and reducing circulation time.^{274, 275} Thus, early development efforts required comprehensive analysis of critical nanoparticle quality attributes. This previous work showed that silk nanoparticles and other silk fibroin-capped nanoparticles possess enhanced colloidal stability across broad ranges of pH and ionic strength due to steric hindrance effects. Moreover, surface modification of silk nanoparticles with cationic polymers improved their colloidal stability in biological media.^{97, 276}

However, the effect of counterions on the physicochemical stability of silk nanoparticles remains poorly understood. Studies on potassium chloride-loaded alginate beads, a model study for counterions interaction, have shown that calcium promotes stable crosslinking, while potassium affects entrapment and gel structure.²⁷⁷ These findings highlight the importance of ion interactions and the need to explore their role in silk nanoparticle stability.

The observation of a significant impact of cations on silk structure during silk nanoparticle formation led us to speculate that cation-induced structural changes may directly influence the functionality and stability of silk. Due to poor reporting on the long-term stability of silk nanoparticles system without stabilizing agent—despite some data on short-term

stability (~24 h)⁹⁷—I conducted further investigations into the physical stability of cation-mixed silk nanoparticles using electrical asymmetrical flow-field flow fractionation (EAF4), a novel separation based approach that has gained attention in the nanoscale separation and analysis of pharmaceuticals in recent years. AF4 is a subtechnique of flow field-flow fractionation (FFF) and involves the application of a hydrodynamic force (cross flow) to the elution flow. Under parabolic flow in the FFF channel, analyte velocities vary based on their unique physicochemical properties, leading to effective fractionation with different elution times.¹³⁹⁻¹⁴² EAF4 incorporates an additional electrical field from electrodes to enhance the separation of charged analytes with different electrophoretic mobilities and diffusion coefficients.¹⁴¹

In the present study, I provide the first report of the physical stability of silk nanoparticles prepared by antisolvent precipitation in the presence of Ca²⁺ and K⁺ cations. Using an EAF4-multidetector setup and orthogonal analytical techniques, I report the short-term and long-term storage stability of these silk nanoparticles and provide new insights into their physicochemical properties.

4.2. Materials and Methods

4.2.1. Materials

All reagents and solvents were purchased from Sigma-Aldrich or Acros Organics (>98% purity) and used without additional purification unless noted. *B. mori* silk cocoons were purchased from Scientific Center on Sericulture, Vratsa, Bulgaria.

4.2.2. Aqueous Silk Fibroin Preparation

Silk fibroin solution was prepared from *Bombyx mori* cocoons by degumming and dissolution, using a method described elsewhere.²³ For the purpose of this article, I use the term “silk” to refer to silk fibroin.

Dried degummed silk was dissolved in LiBr at 60 °C for 4 h under gentle stirring and dialyzed against deionized water using a 3.5K MWCO dialysis cassette (Slide-A-Lyzer 3.5K Dialysis Cassette G2, Thermo Scientific, Rockford, IL, USA) for 48 h at ambient temperature. The silk solution was collected following centrifugation (2,885g, 40 min at 4 °C) (PK 121R Centrifuge, rotor T515, ACL International Srl, Milan, Italy). Silk concentration (% w/v) was calculated from the dried weight of silk obtained by incubating 500 µL aliquots at 60 °C for 24 h.

4.2.3. Silk Nanoparticle Manufacture

Cation-coordinated silk nanoparticles were assembled following a method reported elsewhere.²³ Briefly, a cation-mixed silk solution was prepared to yield 3% w/v silk supplemented with CaCl₂ (Ca²⁺:silk; 0.7:1 and 11.5:1 mg/g) and KCl (K⁺:silk; 1.1:1 and 17.3:1 mg/g) as these concentrations exhibited optimal physicochemical properties,^{245, 278} and closely reflected the mass ratio in the silkworm middle silk gland.^{17, 28, 30} Then, 6 mL of the mixture was dropped from a height 7.5 cm above the meniscus into 30 mL of isopropanol at a syringe pump speed of 1 mL/min and a stirring rate of 400 rpm. The silk nanoparticles were centrifuged at 48,400g for 2 h at 4 °C, and sonicated twice at 30% amplitude for 30 s in deionized water. This washing and centrifugation cycle was repeated twice more. Silk nanoparticles were manufactured from three independent silk batches.

4.2.4. Temporal Physical Stability Evaluation of Silk Nanoparticles

Short-term colloidal stability of silk nanoparticles was evaluated prior to long-term storage (56 days) by storing silk nanoparticles in the 0.0625 to 1 mg/mL concentration range, dispersed in deionized water, sodium phosphate buffer (10 mM, pH 7.4), 1× phosphate-buffered saline (pH 7.0–7.2) (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄ pH 7.0–7.2), and 0.9% w/v NaCl (normal saline). The impact of pH (pH 5.8, 7.4, and 8.0) was also assessed in 10 mM sodium phosphate buffer. Diffusion coefficient, particle size, and polydispersity index (PDI) were measured to evaluate colloidal stability under each condition by dynamic light scattering (DLS), following a 2 h incubation at ambient temperature (**Figure 4.1a**).

For long-term stability, the solution phase stability of the dispersed silk nanoparticles was determined in deionized water and 10 mM sodium phosphate buffer (pH 7.4). Aliquots of 1 mg/mL silk nanoparticle suspension were prepared in each solution at 4 °C. Their physicochemical properties were tracked by dynamic light scattering (DLS), electrophoretic light scattering (ELS), nanoparticle tracking analysis (NTA), electrical asymmetric flow field-flow fractionation (EAF4), and field emission scanning electron microscopy (FE-SEM). The analytical workflow, including the sampling timepoints, is presented in **Figure 4.1b**.

4.2.5. Size and zeta potential determination

Particle size (Z-average) and PDI were measured using a Zetasizer Nano ZS (Malvern Panalytical, Worcestershire, UK) with a 633 nm helium-neon laser and a 173° detection angle (non-invasive back scattering). All measurements were performed using a 1:25 ratio (v/v) of silk nanoparticles: (i) 0.0625–1 mg/mL for short-term storage and (ii) 1 mg/mL for long-term storage to deionized water at 25 °C, with a count rate of 203.6 kcps, a measurement duration of 70 s, and measurement position at 3 mm. The diffusion self-interaction parameter, k_D , was

determined by measuring the diffusion coefficient of silk nanoparticle samples as a function of concentration (0.0625–1 mg/mL).

Zeta potential was measured using electrophoretic light scattering (Zetasizer Nano-ZS Malvern Instrument) and a 1:20 ratio (v/v) of long-term storage silk nanoparticle suspension (1 mg/mL) to deionized water at 25 °C, a count rate of 161.5 kcps, and a measurement position of 2 mm. Each silk nanoparticle type was analyzed as three independent replicates consisting of five technical replicates for DLS and three technical replicates for ELS.

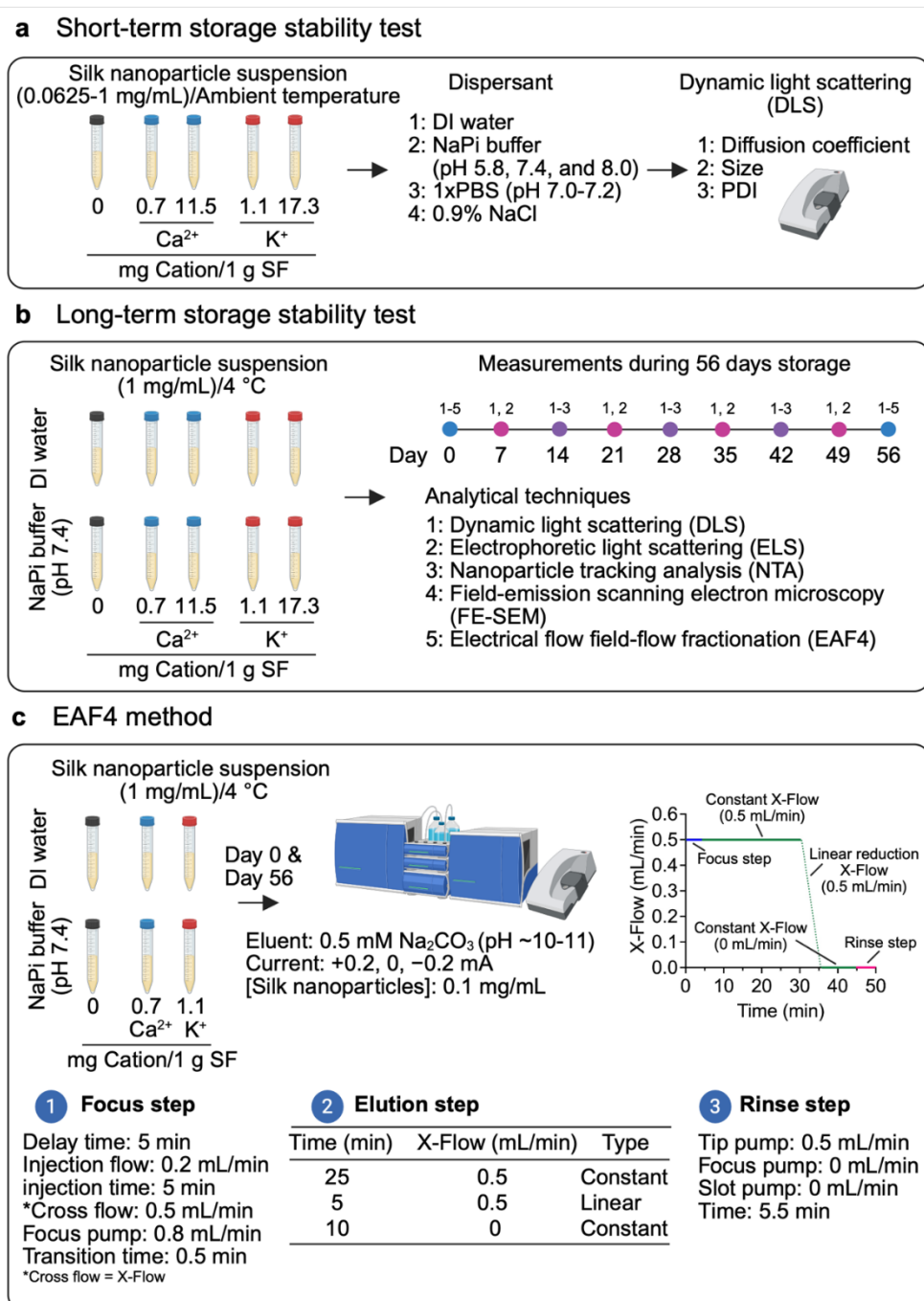


Figure 4.1 Schematic of silk nanoparticle storage stability study illustrating measurement workflow and analytical techniques, performed (a) after 2 h incubation at ambient temperature, (b) over 56 days, and (c) the electrical flow field-flow fractionation (EAF4) method used for nanoparticle characterization at day 0 and day 56. Abbreviations: deionized water (DI water); sodium phosphate buffer (NaPi buffer).

4.2.6. Nanoparticle Tracking Analysis (NTA)

At each stability time point (**Figure 4.1b**), the silk nanoparticle suspension (1 mg/mL) was aliquoted, and diluted in Milli-Q water to achieve 20–80 particles per frame during NTA data acquisition. The corresponding particle size and concentration were measured alternate weekly using Nanoparticle Tracking Analysis (NTA 3.4 Build 3.4.003, Malvern Panalytical Ltd., Worcestershire, UK) configured with a sCMOS camera, a 488 nm laser, and a syringe pump set at 100 (Harvard Apparatus Model 98-4730 Syringe Pump, Massachusetts, US).

Measurements were acquired at ambient temperature and recorded for five replicate videos of 60 s duration. Capture settings included a camera level of 9 for the smaller-sized silk nanoparticle samples (0 mg Cation, 0.7 mg Ca²⁺, and 1.1 mg K⁺) and a camera level of 8 for the larger nanoparticle samples (11.5 mg Ca²⁺ and 17.3 mg K⁺). Data were processed using NTA 3.4 (version 3.4.003) software with a detection threshold of 6.²⁵⁰ Each silk nanoparticle type was analyzed as three independent replicates consisting of five technical replicates.

4.2.7. Analysis of Silk Nanoparticle Morphology and Size by Field Emission Scanning Electron Microscopy (FE-SEM)

Silk nanoparticle suspensions were aliquoted on days 0 and 56 and gently vortexed to achieve resuspension. A droplet of nanoparticle suspension was transferred onto a 5×5 mm silicon wafer chip (Ted Pella, Inc., CA, USA), dried at ambient temperature for 24 h, and sputter-coated with gold from a height of 35 mm for 40 s at 0.08 mb and 30 mA (Agar Scientific Manual Sputter Coater, Agar Scientific Ltd., Essex, UK). The silk nanoparticles were imaged by FE-SEM at 20,000× and 60,000× magnifications at 5 kV (Hitachi SU6600, Hitachi High-Tech Europe GmbH, Krefeld, Germany).

4.2.8. Analysis of Silk Nanoparticle Physicochemical Properties by Multidetector

Electrical Asymmetric Flow Field-Flow Fractionation (EAF4)

Silk nanoparticles were separated, and particle stability was tested at days 0 and 56 using an EAF2000 Asymmetrical Flow FFF system (AF2000, PostNova Analytics, Landsberg am Lech, Germany), controlled by an electrical module (#PN2411, Postnova Analytics, Landsberg am Lech, Germany), and equipped with a conductivity meter. The spacer thickness was 350 μm , and a regenerated cellulose membrane (modified RC membrane) with a molecular weight cut-off of 10 kDa (Postnova AF2000 MF-AF4 Analytic RC Membrane, Z-AF4-MEM-618-10kDa, Postnova Analytics, Landsberg am Lech, Germany) was used as the accumulation wall for all measurements. The in-line detectors multiplexed with EAF4 were a 21-multi-angle light scattering (MALS) detector (#PN3621, Postnova Analytics), a refractive index (RI) detector (#PN3150, Postnova Analytics), a UV detector (#SPD-M40, Postnova Analytics), and an online Zetasizer Nano ZS (Malvern Panalytical).

EAF4 separation and inline analysis were performed on days 0 and 56 for silk nanoparticles suspended in deionized water (0.1 mg/mL). The EAF4 method was as described elsewhere²⁷⁹ with minor modifications and included an injection volume of 20 μL , a 0.5 mM Na_2CO_3 (~pH 10) carrier solution (0.1 μm pore size filtered), and an applied current of ± 0.2 mA (**Figure 4.1c**). The term “AF4” used in this study, from this point onward, refers to the EAF4 data analyzed under a neutral current (0 mA) only.

The nanoparticle shape factor was determined according to the radius of gyration (R_g) derived from MALS and the hydrodynamic radius (R_h) from inline DLS measurements.

$$\text{Shape factor} = \frac{R_g}{R_h} \quad \text{Equation 4.1}$$

Electrophoretic mobility (μ) and zeta potential (ζ) were evaluated using the NovaFFF-EAF4 Software Module (Postnova Analytics). The μ (mm cm/Vs) was calculated as a function

of the correlation between the drift velocity induced by the electrical field (n_{em}) and electrical field strength (E) using **Equation 4.2–4.3**.

$$\mu = \frac{n_{em}}{E} \quad \text{Equation 4.2}$$

$$n_{em} = n - n_c \quad \text{Equation 4.3}$$

where n is the total drift velocity and n_c the drift velocity induced by the cross flow.

$$E = \frac{I}{Ak} \quad \text{Equation 4.4}$$

where I is the applied current, A is the channel area, and k is the conductivity.

Zeta potential (mV) was estimated using an input parameter of standard nanoparticles with Smoluchowski approximation $f(ka)$ of 1.5 (**Equation 4.5**).

$$z = \left[\frac{3}{2} \times \frac{\eta\mu}{\epsilon_0\epsilon_r f(ka)} \right] \times 1000 \quad \text{Equation 4.5}$$

where η is viscosity at 25 °C of eluent (Pa s), ϵ_0 the vacuum permittivity (physical constant) ($\sim 8.854187 \times 10^{-12} \text{ AsV}^{-1}\text{m}^{-1}$), ϵ_r is the relative permittivity of water at 25 °C (~ 78.53114), μ is calculated electrophoretic mobility (mm cm/Vs), $f(ka)$ is Henry's function, k is the reciprocal of Debye length, R_h is the hydrodynamic radius, and ka is the estimated ratio of the particle radius to the electrical double layer.

4.2.9. Statistical Analyses and Software

GraphPad Prism 9 (GraphPad Software, Boston, MA, USA) was used for the graphs and statistical analyses, including two-way ANOVA, Dunnett's multiple comparisons test, and Šídák's multiple comparisons test. Asterisks denote statistical significance, experimental repeats (n) are specified in each figure legend, and all data are presented as mean values $\pm$ (SD). The cartoon illustration was prepared using Microsoft PowerPoint version 16.93 (Microsoft® PowerPoint for Mac, Redmond, WA, USA) and BioRender (Scientific Image and Illustration Software, <https://www.biorender.com>). All AF4 and EAF4 data were analyzed using NovaFFF AF2000 Software (Postnova Analytics).

4.3. Results

4.3.1. Colloidal Stability of Silk Nanoparticles

The colloidal stability of the silk nanoparticles was investigated as a function of concentration, formulation buffer composition, and pH, following a 2-hour incubation at ambient temperature (**Figure 4.2**). The silk nanoparticles exhibited high physical stability in deionized water at 2 h, with minimal alterations in the diffusion coefficient, particle size, and PDI. Only minor changes in size were observed for nanoparticles dispersed in sodium phosphate buffer (10 mM, pH 7.4). However, the particle diameter increased over the tested concentration range. In the absence of cations, the silk nanoparticles had a diameter in the 79–135 nm range, while nanoparticles prepared with 0.7 mg Ca^{2+} exhibited diameters in the 87–132 nm range, and nanoparticles formulated with 1.1 mg K^{+} ranged from 86–123 nm in diameter. In contrast, silk nanoparticles formulated with higher amounts of Ca^{2+} (11.5 mg) and K^{+} (17.3 mg) were approximately 255 nm and 276 nm in diameter, respectively, and were relatively stable, exhibiting only minimal changes in particle diameter. Increases in particle diameter and PDI changes were also seen for nanoparticles dispersed in phosphate-buffered saline (pH 7.0–7.2) and 0.9% w/v NaCl, resulting in micron-scale particle complex formation (up to $\sim 3.6 \text{ d.}\mu\text{m}$) (**Figure 4.2a**). Under different pH conditions (pH 5.8, 7.4, and 8.0), particle growth was observed—specifically for the low mass ratio ions, with an increase in particle size and PDI within a 2 h incubation time. In contrast, no significant difference was observed in particle growth for silk nanoparticles formulated with high mass ratio ions, as well as negligible changes in PDI value (**Figure 4.2b**). The diffusion self-interaction parameter (k_D), derived from the correlation between the particle concentration and diffusion coefficient, is shown in **Table 4.1**.

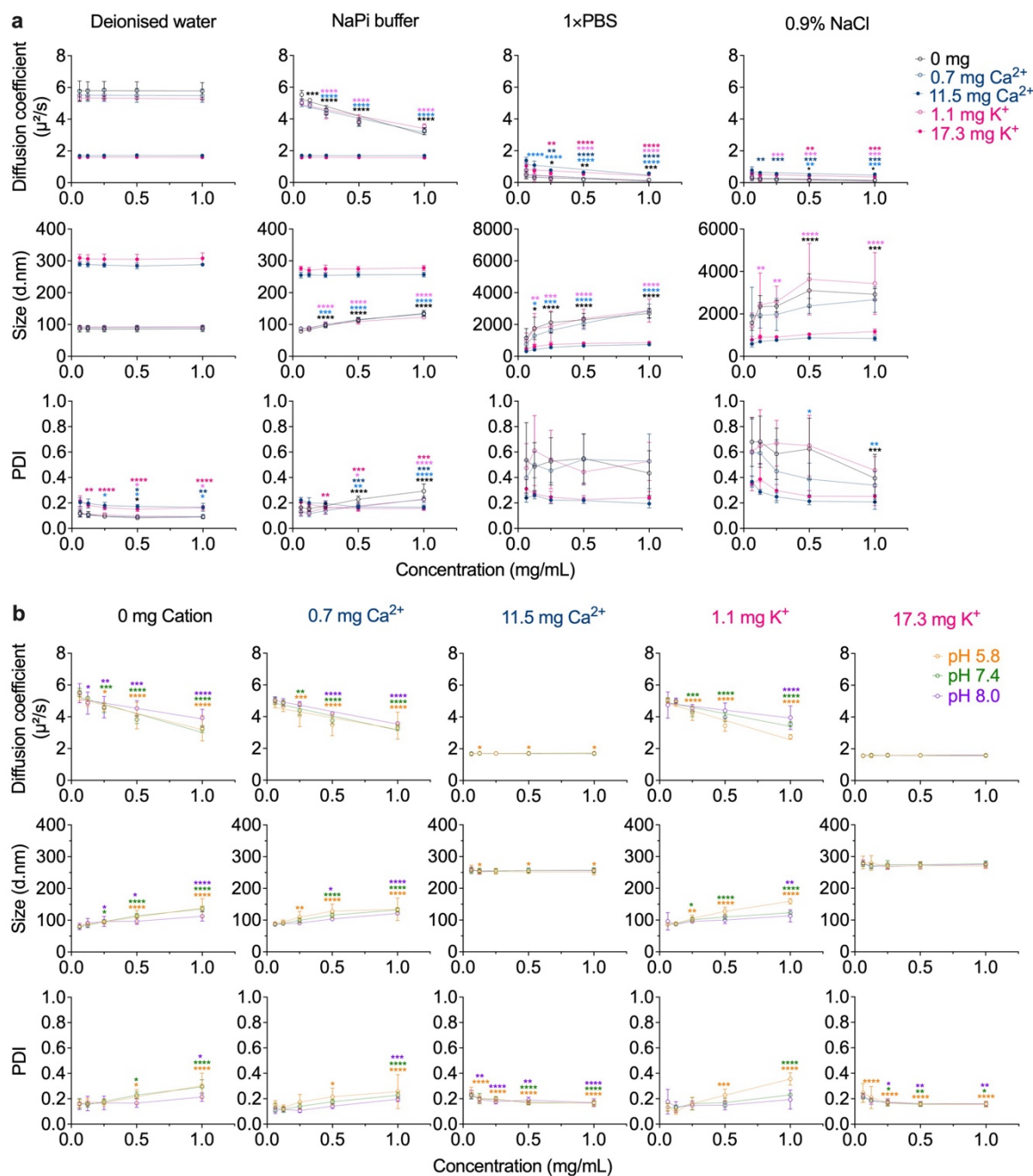


Figure 4.2 Colloidal stability of silk nanoparticles. Diffusion coefficients, particle sizes, and polydispersity indexes (PDIs) were determined by dynamic light scattering (DLS) as a function of nanoparticle concentration. **(a)** The impact of continuous phase, which was either deionized water (DI water), 10 mM sodium phosphate (NaPi) buffer (pH 7.4), 1× phosphate-buffered saline (PBS) (pH 7.0–7.2), or 0.9% w/v NaCl, on nanoparticle agglomeration behavior ($n = 3$). **(b)** Agglomeration behavior of silk nanoparticles as a function of nanoparticle concentration and pH (5.8–8.0) was assessed in 10 mM NaPi buffer ($n = 3$). All conditions were measured after 2-hour incubation at ambient temperature. Two-way ANOVA and Dunnett's multiple comparisons test were used in the statistical analysis, using 0.0625 mg/mL as a control baseline: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Colored asterisks represent statistical analyses for each sample and condition; 0 mg Cation (control, black), 0.7 mg Ca^{2+} (light blue), 11.5 mg Ca^{2+} (blue), 1.1 mg K^+ (light pink), 17.3 mg K^+ (pink), pH 5.8 (orange), pH 7.4 (green), pH 8.0 (purple). The experiments were conducted as three independent formulation batches with three technical repeat each ($n = 3$).

Table 4.1 Measured diffusion self-interaction parameter (k_D) of silk nanoparticles in different dispersants ($n = 3$). Abbreviations: deionized water (DI water); phosphate buffered saline (PBS); sodium phosphate buffer (NaPi buffer).

Name	DI water	NaPi buffer (10 mM)			1×PBS (pH 7.0–7.2)	NaCl (0.9% w/v)
		pH 5.8	pH 7.4	pH 8.0		
0 mg Cation	−0.014	−2.063	−2.373	−1.335	−0.237	−0.103
0.7 mg Ca^{2+}	−0.030	−1.467	−1.819	−1.592	−0.440	−0.155
11.5 mg Ca^{2+}	0.010	0.047	−0.010	−0.006	−0.714	−0.206
1.1 mg K^+	−0.065	−2.442	−1.597	−0.985	−0.389	−0.220
17.3 mg K^+	0.001	0.044	−0.025	−0.002	−0.414	−0.176

4.3.2. Physicochemical Characteristics of Silk Nanoparticles in Deionized Water and Sodium Phosphate Buffer

At physiological pH (pH 7.4), silk nanoparticles exhibited different agglomeration behaviours, with 0 mg cation nanoparticles forming large agglomerates (lowest negative k_D value). Nanoparticles formulated in the presence of 0.7 mg Ca^{2+} and 1.1 mg K^+ also formed larger aggregates in comparison to nanoparticles formulated with 11.5 mg Ca^{2+} and 17.3 mg K^+ that were stable. Negligible changes in agglomeration were observed in response to pH among the silk nanoparticles; however, particle agglomeration was greater at pH 5.8 than at pH 8.0 (**Figure 4.2b** and **Table 4.1**).

Silk nanoparticle stability at 4 °C in deionized water and sodium phosphate buffer was studied using multiple orthogonal analytical techniques (**Figure 4.3** and **Figure 4.4**). Nanoparticle suspension stability was initially measured using DLS and NTA, which demonstrated no significant changes in measured particle size or PDI over 56 days of storage in deionized water—especially for smaller particles (**Figure 4.3a**). In contrast, significant agglomeration was observed for silk nanoparticles dispersed in sodium phosphate buffer, particularly in the case of nanoparticles with a smaller baseline particle size (i.e., 0 mg Cation, 0.7 mg Ca^{2+} , and 1.1 mg K^+), which showed a 3- to 4-fold increase in particle size at day 56. A 6- to 12-fold decrease in particle concentration was measured for nanoparticles dispersed in

sodium phosphate buffer (pH 7.4) over 56 days of storage. Negligible changes in nanoparticle surface charge were observed, demonstrating stable zeta potential (ZP) throughout storage (**Figure 4.3a** and **Figure 4.4a**).

Observations from FE-SEM micrographs were consistent with the DLS and NTA size data and confirmed the formation of nanoparticle agglomerates after 56 days of storage at 4 °C in sodium phosphate buffer, but not in deionized water (**Figure 4.3b** and **Figure 4.4b**). The impact of temperature on silk nanoparticle stability was profiled at ambient (25 °C) and physiologically relevant temperatures (37 °C), showing that storage at 37 °C for control and 17.3 mg K⁺ nanoparticles, resulted in the formation of micron-sized silk nanoparticle agglomerates (~5 µm) (**Figure 4.5**).

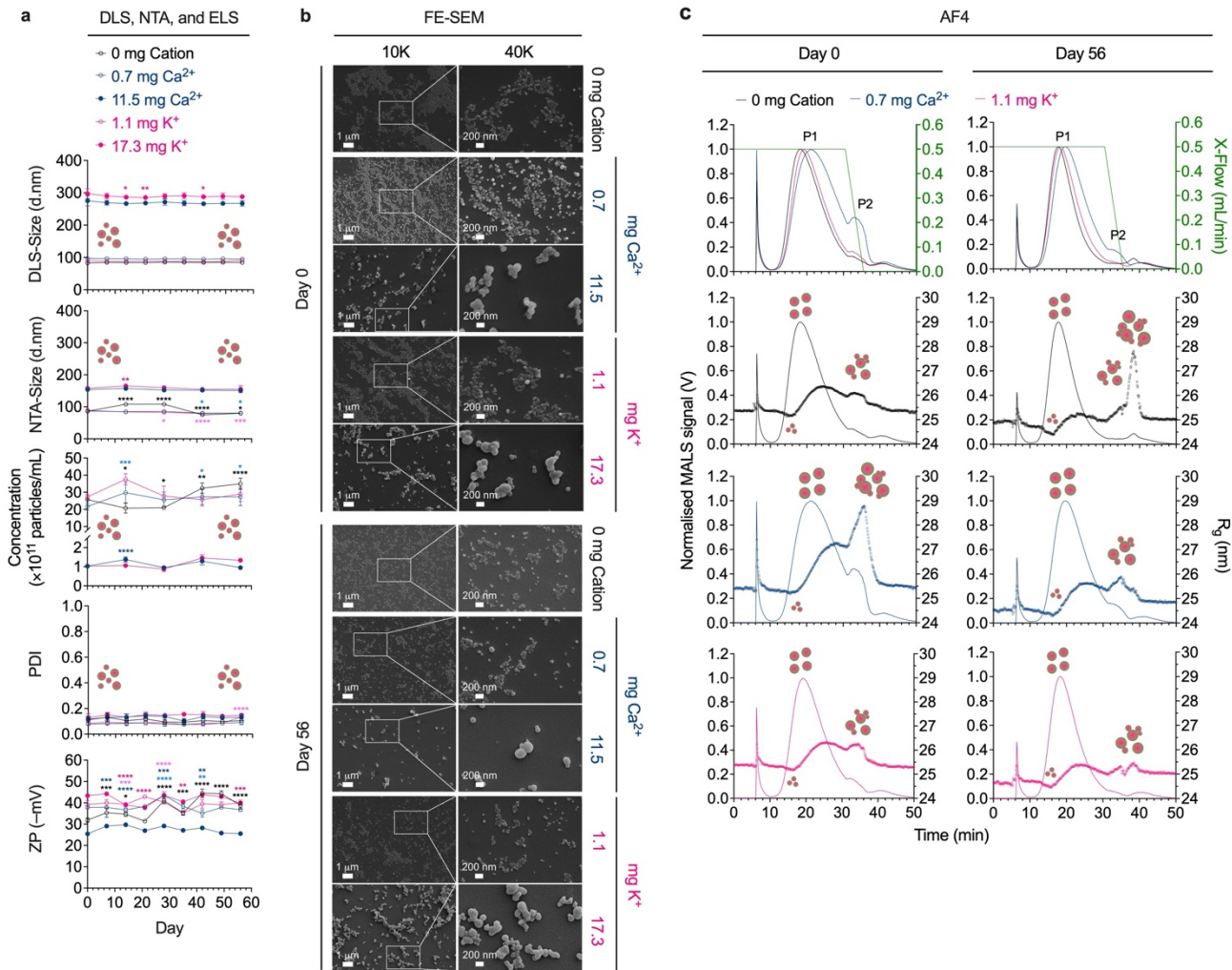


Figure 4.3 Storage stability of silk nanoparticles in deionized water at 4 °C. (a) Particle size, concentration, polydispersity index (PDI), and zeta potential (ZP) measured over 56 storage days in deionized (DI) water at 4 °C ($n = 3$). (b) SEM images (10,000 $\times$ and 40,000 $\times$ magnifications) of silk nanoparticles dispersed in deionized water at 4 °C on day 0 and day 56 ($n = 1$). (c) The fractograms of silk nanoparticles obtained from the conventional asymmetric flow field-flow fractionation (AF4) (no applied current; 0 mA) ($n = 3$). Two-way ANOVA and Dunnett's multiple comparisons test were used in statistical analysis, comparing the impact of storage day to day 0; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). All experiments were conducted as three independent experimental repeats with three technical repeats each, except SEM and AF4 that were conducted as three technical repeats ($n = 3$). Different colored asterisks represent statistical analysis for each sample; 0 mg (control, black trace), 0.7 mg Ca^{2+} (light blue trace), 11.5 mg Ca^{2+} (blue trace), 1.1 mg K^+ (light pink trace), 17.3 mg K^+ (pink trace).

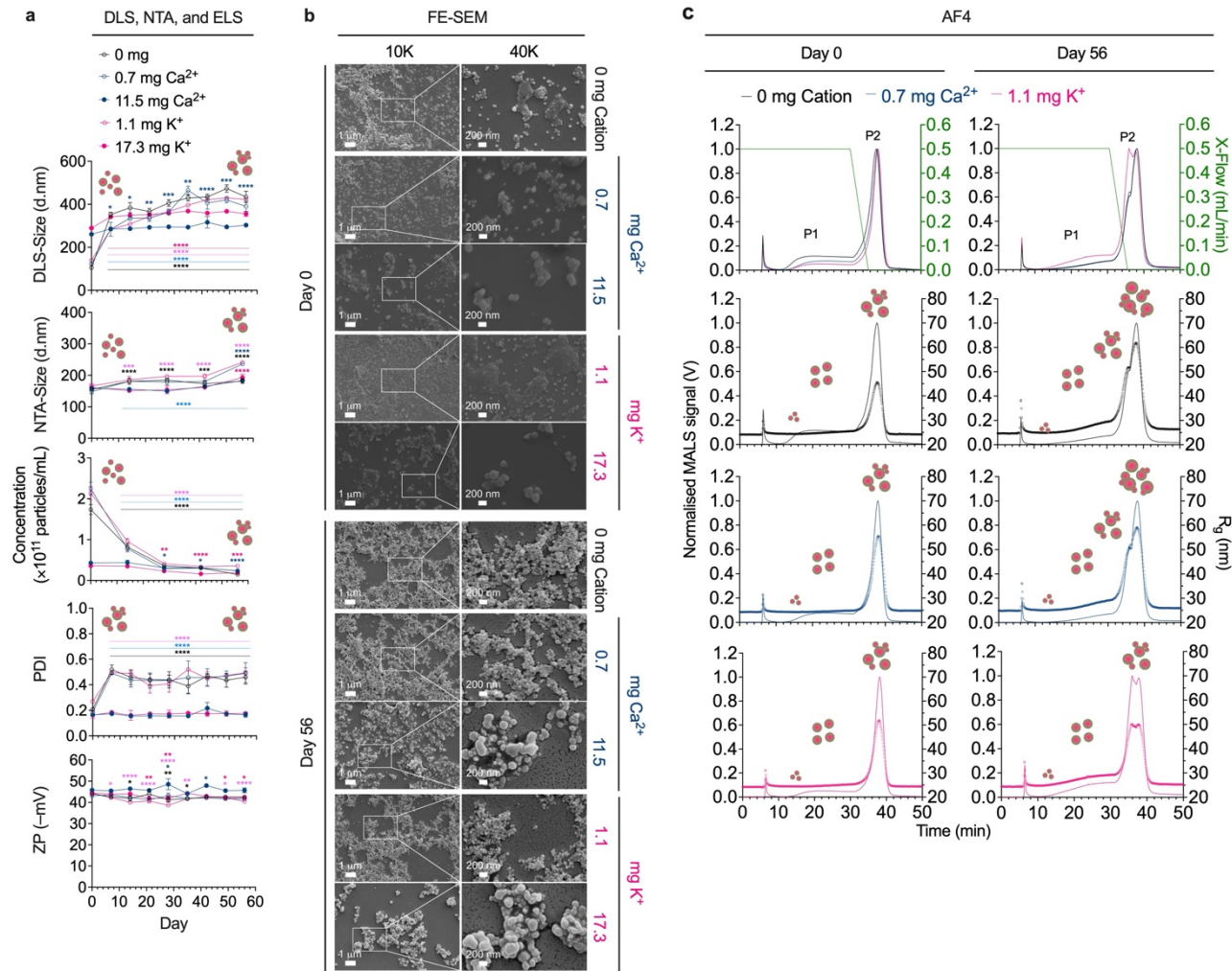


Figure 4.4 Storage stability of silk nanoparticles in sodium phosphate (NaPi) buffer (pH 7.4) at 4 °C. (a) Particle size, concentration, polydispersity index (PDI), and zeta potential (ZP) measured over 56 storage days in 10 mM NaPi buffer (pH 7.4) at 4 °C ($n = 3$). (b) FE-SEM micrographs (10,000 $\times$ and 40,000 $\times$ magnifications) of silk nanoparticles dispersed in 10 mM NaPi buffer (pH 7.4) at 4 °C on day 0 and day 56 ($n = 1$). (c) Corresponding fractograms of nanoparticles obtained from conventional asymmetric flow field-flow fractionation (AF4) ($n = 3$). Two-way ANOVA and Dunnett's multiple comparisons test were used in statistical analysis, comparing the impact of storage duration; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). All experiments were conducted as three independent experimental repeats with three technical repeats each, except SEM and AF4 that were conducted as three technical repeats ($n = 3$). Different color of an asterisk represents statistical analysis for each sample; black: 0 mg Cation, light blue: 0.7 mg Ca^{2+} , blue: 11.5 mg Ca^{2+} , light pink: 1.1 mg K^+ , pink: 17.3 mg K^+ .

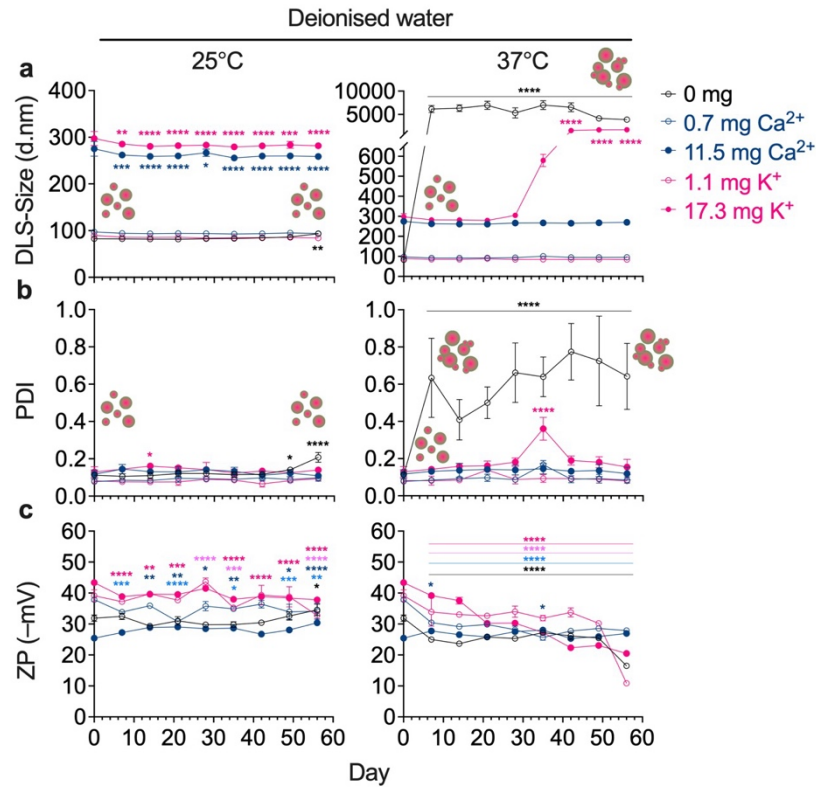


Figure 4.5 Time storage stability of silk nanoparticles (SNPs) in water. (a) Particle size as measured by Dynamic Light Scattering: DLS, (b) particle concentration, (c) polydispersity index (PDI), and (d) zeta potential (ZP) measured over 56 storage days in deionized water at 25 °C and 37 °C. Two-way ANOVA and Dunnett's multiple comparisons test were assessed for statistical analyses comparable to day 0: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). All experiments were conducted as three independent experimental repeats with three technical repeats each ($n = 3$).

In addition, I also conducted a preliminary study using a biologically relevant system, DMEM supplemented with 10% FBS, following 2 and 24 h incubation at 37 °C. The result demonstrated the changes in surface environment, resulting in a reduction in ZP to approximately -24 mV for all samples, compared to their ZP in deionized water (Day 0); however, no significant difference between 2 and 24 h incubation. For the size property, most samples showed no significant differences between 2 and 24 hours, with sizes remaining within the range reported in deionized water, while the PDI increased above 0.2 (**Figure 4.6**). Furthermore, the thioflavin T assay showed that Ca^{2+} and K^{+}

significantly increase thioflavin T entrapment into particles, while antisolvent precipitation occurs (Figure 4.7).

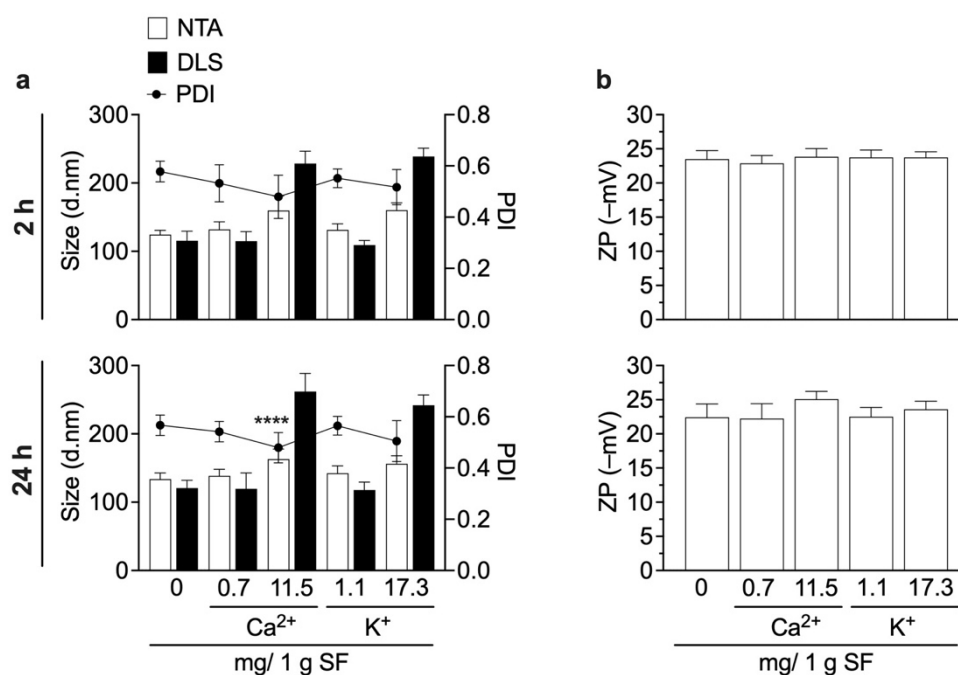


Figure 4.6 Silk nanoparticle size and zeta potential in a biological-relevant system. Silk nanoparticles (1 mg/mL) were dispersed in complete media (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v FBS, 50 U/mL penicillin, and 50 mg/mL streptomycin), following by incubation at 37 °C for 2 and 24 h: **(a)** particle size via Dynamic light scattering (DLS) and Nanoparticle tracking analysis (NTA), and **(b)** zeta potential (ZP) via Electrophoretic light scattering (ELS). Two-way ANOVA and Šidák's multiple comparisons test were assessed for statistical analyses comparable to a 2-h incubation: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). All experiments were conducted as three independent experimental repeats with three technical repeats each ($n = 3$).

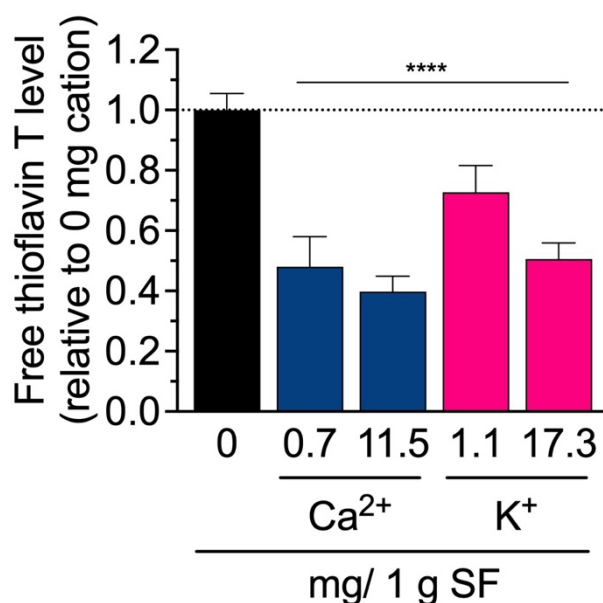


Figure 4.7 Thioflavin T assay. Silk nanoparticle was fabricated by the anti-solvent precipitation method in a semi-batch with 3% w/v silk fibroin (SF) solution containing 100 mL of thioflavin T (1 mM). The reduction of free thioflavin T molecule was measured in a supernatant collected after centrifugation at 48,000g for 2 h, followed by a fluorescence reading at 460 nm (ex. 355 nm). One-way ANOVA and Dunnett's multiple comparisons test were assessed for statistical analyses comparable to a baseline 0 mg cation (dot line): $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). The experiment was conducted as three independent experimental repeats with three technical repeats each ($n = 3$).

4.3.3. Analysis of Silk Nanoparticle Physical Stability at Different Timepoints Using

Asymmetric Flow Field-Flow Fractionation with online UV-Multiangle Light Scattering, and Dynamic Light Scattering (AF4-UV-MALS-DLS)

Asymmetric flow field-flow fractionation (AF4) was used to investigate the particle size distribution profile, interparticle interactions, and the shape factor of silk nanoparticles during storage.

When stored in deionized water, silk nanoparticles formulated with 0.7 mg Ca²⁺ and 1.1 mg K⁺ exhibited a shift in their main elution peak (P1) relative to the 0 mg nanoparticles, from 18.1 min to 21.0 and 19.1 min, respectively. This indicated a larger size, with an increase in R_g from 25.5 nm to 26.1 nm and 25.6 nm, respectively. Interestingly, the 0.7 mg Ca²⁺ nanoparticles exhibited a higher MALS signal at ~33 min compared to the other silk nanoparticles, indicating the presence of a larger number of larger particles and/or aggregates at peak 2 (P2) with R_g at 28.0 nm. However, control silk nanoparticles (0 mg cation) and 1.1 mg K⁺ silk nanoparticles had smaller R_g at 26.1 nm for both. After

dispersal for 56 days in deionized water, the RT of P1 was slightly shorter than at day 0 (R_g in parentheses), appearing at 17.7 min, 19.6 min, and 18.3 min for 0 mg cation nanoparticles (24.7 nm), 0.7 mg Ca^{2+} silk nanoparticles (24.8 nm), and 1.1 mg K^+ nanoparticles (24.9 nm), respectively. A noticeable shift in the RT of P2 was observed in the 0 mg Cation silk nanoparticles (27.8 nm), increasing from 32.7 min to 38.4 min, whereas no significant changes in RT for P2 were observed in the other samples (~ 33 min). Interestingly, the R_g values for 0.7 mg Ca^{2+} silk nanoparticles and 1.1 mg K^+ silk nanoparticles were smaller at 25.6 nm and 25.0 nm, respectively (**Figure 4.3c** and **Table 4.2**). After storage for 56 days, slight changes in the percentage recovery were evident in the 0 mg Cation nanoparticles (85.79% to 82.99%) and 0.7 mg Ca^{2+} nanoparticles (92.94% to 75.30%). In contrast, the 1.1 mg K^+ nanoparticles showed a preferable recovery percentage, at 92.61% to 95.51%, until 56 days of storage (**Figure 4.8a**).

DLS measurement of the particle size for the silk nanoparticles dispersed in 0.5 mM Na_2CO_3 ($\sim \text{pH } 10$) for 1 h, which mimics the duration of the AF4 analysis, showed particle growth compared to those dispersed in sodium phosphate buffer (pH 8.0), with an increase in Z-average from 121.4 d.nm to 142.8 d.nm for the 0.7 mg Ca^{2+} nanoparticles. In contrast, a negligible increase in Z-average was observed for the 0 mg cation nanoparticles (from 112.3 d.nm to 116.2 d.nm) and the 1.1 mg K^+ nanoparticles (from 113.3 d.nm to 126.7 d.nm) (**Figure 4.9a**). Additionally, the change in zeta potential of 0 mg cation nanoparticles was shifted to a more negative charge (-30 mV to ~ -37 mV), while 0.7 mg Ca^{2+} and 1.1 mg K^+ nanoparticles zeta potential became less negative (-40 mV to ~ -30 mV) (**Figure 4.9b**).

Silk nanoparticles dispersed in sodium phosphate buffer (pH 7.4) demonstrated different fractograms for the 0 mg cation, 0.7 mg Ca^{2+} , and 1.1 mg K^+ silk nanoparticles, which showed a broad peak of small R_g particles sized 24.5 nm at 18.9 min (24.5 nm), 24.6 nm at 22.1 min, and 24.4 nm at 21.5 min, respectively, and a sharp peak of large R_g particles sized 45.5 nm at 25.9 min, 51.9 nm at 26.6 min, and 55.5 nm at 29.0 min (55.5 nm), respectively. After 56 days, the 0.7 mg Ca^{2+} silk

nanoparticles showed significant changes in RT of P1, which shifted to 28.0 min at 26.6 min, whereas P2 showed no significant changes, eluting at 38.0 min with a size of 59.1 nm. However, the RTs for P1 and P2 of the other silk nanoparticles were shifted to 30.3 min (P1 particle size: 29.5 nm) and 38.1 min (P2 size: 61.8 nm) for 0 mg Cation and to 29.0 min (P1 size: 29.0 nm) and 37.8 min (P2 size: 50.0 nm) for 1.1 mg K^+ . Importantly, the R_g of the silk nanoparticles showed no significant difference after 56 days (**Figure 4.4c** and **Table 4.3**). Similarly to particles dispersed in water, the 0.7 mg Ca^{2+} and 1.1 mg K^+ silk nanoparticles dispersed in sodium phosphate buffer (pH 7.4) exhibited > 82% recoveries from day 0 until day 56, while the 0 mg Cation silk nanoparticles showed recoveries of approximately 70% (**Figure 4.8b**).

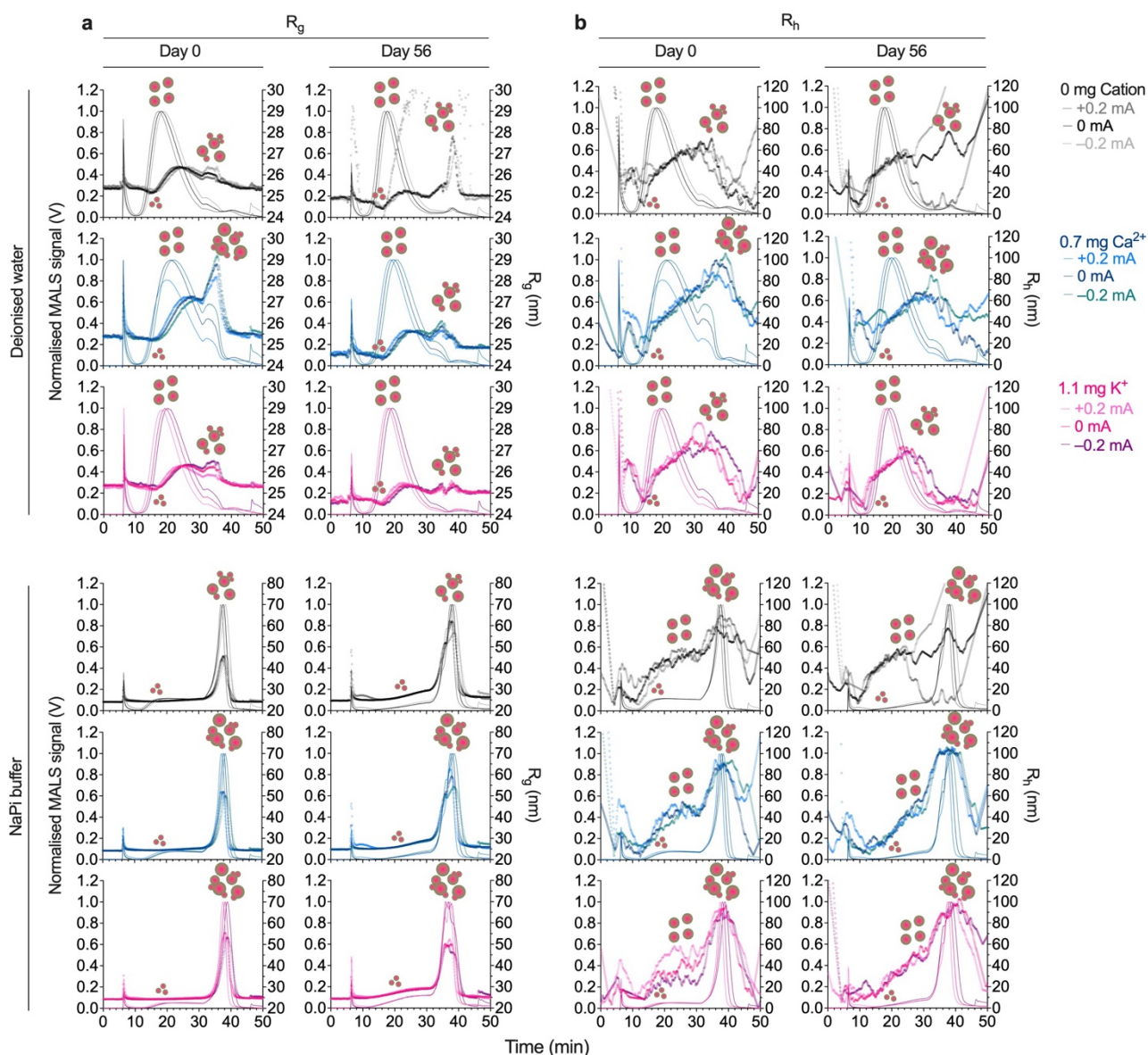


Figure 4.8 Electrical asymmetric flow field-flow fractionation (EAF4) fractograms of silk nanoparticles (SNPs): 0 mg Cation SNP (black), 0.7 mg Ca^{2+} SNP (blue), and 1.1 mg K^+ SNP (pink). The 0.1 mg/mL SNPs were separated in 0.5 mM sodium carbonate under electrical fields with applied positive (+0.2 mA), neutral (0 mA), and negative (-0.2 mA) currents. The SNP EAF4 fractograms for days 0 and 56 of storage in deionized water and 10 mM Sodium phosphate (NaPi) buffer (pH 7.4) at 4 °C were as a correlation of normalized MALS signal together with (a) radius of gyration (R_g) and (b) hydrodynamic radius (R_h). The experiment was conducted as three technical repeats ($n = 3$).

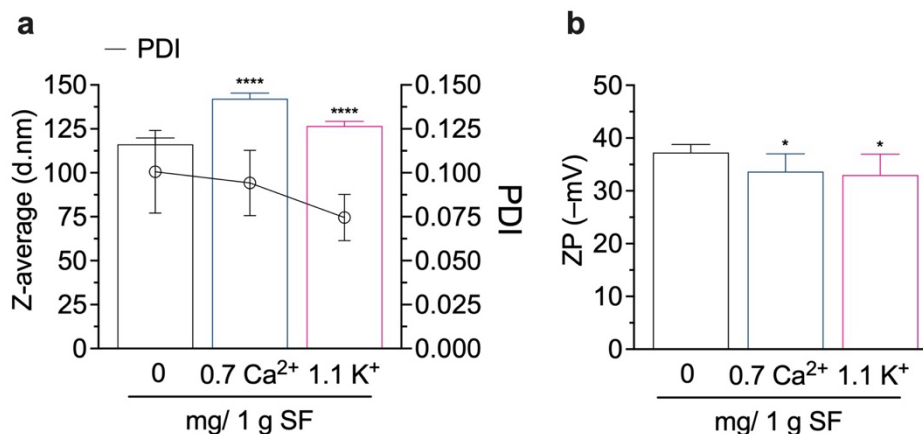


Figure 4.9 Physicochemical properties of silk nanoparticles (SNPs) dispersed in 0.5 mM Na₂CO₃ for 1 h: (a) particle size and PDI analysis (*via* dynamic light scattering; DLS); (b) ZP (*via* electrophoretic light scattering; ELS). Two-way ANOVA, One-way ANOVA, and Dunnett's multiple comparisons test were assessed for statistical analyses comparable to 0 mg cation: $p < 0.05$ (*), $p < 0.01$ (), $p < 0.001$ (***), and $p < 0.0001$ (****). All experiments were conducted as three independent experimental repeats with three technical repeats each ($n = 3$).**

4.3.4. Evaluation of Silk Nanoparticle Surface Charges Using Electrical Asymmetric Flow

Field-Flow Fractionation (EAF4-UV-MALS-DLS)

In deionized water, a shift of P1 that deviated from the neutral current condition was evident for all silk nanoparticles: 0 mg Cation nanoparticles: 18.1 min to 17.3 min (+0.2 mA) and 18.9 min (−0.2 mA); 0.7 mg Ca²⁺ nanoparticles: 21.0 min to 20.1 min (+0.2 mA) and 22.0 min (−0.2 mA); and 1.1 mg K⁺ nanoparticles: 19.1 min to 18.0 min (+0.2 mA) and 20.1 min (−0.2 mA). However, only negligible changes were seen in the RT in response to the electrical field for P2 (32.5–33.3 min) (Figure 4.10a and Table 4.2). Furthermore, no significant differences were detected in the computed particle size of P1 ($R_g = 25.5$ – 26.2 nm and $R_h = 36.7$ – 46.5 nm) under the different applied currents. The 0.7 mg Ca²⁺ silk nanoparticles showed noticeable similar properties to those observed in the absence of an electric field, with agglomerates appearing after an elution time of ~33 min (P2), an R_g range of 27.5–28.0 nm, and an R_h range of 71.4–85.0 nm, while the 0 mg Cation and 1.1 mg K⁺ nanoparticles were smaller in size, with corresponding R_g ranges of 25.9–26.4 nm and R_h ranges of 53.8–85.8 nm (Figure 4.8a-b and Table 4.2).

The nanoparticle shape factor was determined using the R_g/R_h ratio, with 0 mg Cation and 1.1 mg K^+ nanoparticles having shape factors of 0.60–0.70, while 0.7 mg Ca^{2+} nanoparticles had a lower shape factor value of 0.56–0.59. Interestingly, a greater deviation from 0.755 was detected for P2, ranging from 0.30 to 0.51 on average (**Figure 4.10a** and **Table 4.2**).

The zeta potential of the nanoparticles was estimated using EAF4-UV-MALS. The 0 mg Cation silk nanoparticles exhibited the lowest zeta potential at -60.30 mV, and no considerable differences were observed between 0.7 mg Ca^{2+} and 1.1 mg K^+ nanoparticles, which showed zeta potentials at -61.24 mV and -61.15 mV, respectively (**Table 4.2**). After 56 days, the zeta potential decreased to -62.02 mV, -54.07 mV, and -47.95 mV for 0 mg Cation, 0.7 mg Ca^{2+} , and 1.1 mg K^+ nanoparticles, respectively. Interestingly, the ELS data indicated less negative zeta potentials for silk nanoparticles dispersed in 0.5 mM Na_2CO_3 ($\sim$ pH 10) for 1 h, at -37.03 mV for 0 mg Cation nanoparticles, -34.33 mV for 0.7 mg Ca^{2+} nanoparticles, and -33.07 mV for 1.1 mg K^+ nanoparticles (**Figure 4.9b**). The percentage recovery of the measured fractions was calculated relative to the direct injection method, with recovery rates of $86(\pm 7)$, $93(\pm 3)$, and $93(\pm 8)\%$ achieved for the 0 mg Cation, 0.7 mg Ca^{2+} , and 1.1 mg K^+ nanoparticles, respectively.

After 56 days of storage, the silk nanoparticles dispersed in deionized water maintained desirable responsiveness under different applied currents, displaying similar shifting profiles in RT for P1; 0 mg Cation nanoparticles: 17.7 min to 16.8 min ($+0.2$ mA) and 18.6 min (-0.2 mA), 0.7 mg Ca^{2+} nanoparticles: 19.6 min to 18.0 min ($+0.2$ mA) and 20.7 min (-0.2 mA), and 1.1 mg K^+ nanoparticles: 18.3 min to 17.6 min ($+0.2$ mA) and 19.3 min (-0.2 mA) and with a negligible shift for P2 (0 mg Cation: 37.7–39.0 min, 0.7 mg Ca^{2+} and 1.1 mg K^+ silk nanoparticles: 32.8–33.7 min). However, most RTs reported on day 56 were significantly different from those on day 0 (**Figure 4.8a-b** and **Table 4.2**). The prominent reduction in R_g was mainly observed in P1 rather than P2, whereas the R_h (in parentheses) fluctuated between $+0.2$ mA and -0.2 mA; 0 mg cation silk nanoparticles (53.8–62.9 nm to 5.5–101.4 nm), 0.7 mg Ca^{2+} nanoparticles (71.4–85.0 nm to 25.9–40.4 nm) and 1.1 mg K^+

nanoparticles (63.9–85.8 nm to 4.0–13.4 nm). These changes in R_g and R_h altered the shape factor profile, increasing the shape factor in P2 significantly: 0 mg cation silk nanoparticles (from 0.45–0.51 to 0.24–5.12), 0.7 mg Ca^{2+} nanoparticles (from 0.33–0.40 to 0.66–1.03), and 1.1 mg K^+ nanoparticles (from 0.30–0.42 to 2.73–5.52). The recovery percentage of silk nanoparticles on day 56 changed only slightly from day 0, with recovery rates of 83($\pm$ 2)%, 75($\pm$ 11)%, and 96($\pm$ 7)% for 0 mg Cation, 0.7 mg Ca^{2+} , and 1.1 mg K^+ nanoparticles, respectively.

In contrast to earlier findings, silk nanoparticles dispersed in NaPi buffer (pH 7.4) exhibited outstanding alterations on the fractograms, revealing a different majority of larger particles eluting as P2. Shifted apical peaks for P1 and P2 in response to the electrical field were also observed. The shifting for P1 for 0 mg Cation nanoparticles was from 18.9 min to 17.7 min (+ 0.2 mA) and 20.2 min (–0.2 mA), for the 0.7 mg Ca^{2+} nanoparticles, it was from 22.1 min to 20.4 min (+0.2 mA) and 23.3 min (–0.2 mA), and for the 1.1 mg K^+ nanoparticles it was from 21.5 min to 20.4 min (+0.2 mA) and 22.7 min (–0.2 mA). The shift in P2 was detected as a longer RT: for 0 mg Cation nanoparticles, it was from 37.7 min to 37.0 min (+ 0.2 mA) and 38.4 min (–0.2 mA), for the 0.7 mg Ca^{2+} nanoparticles, it was from 37.9 min to 37.3 min (+0.2 mA) and 38.5 min (–0.2 mA), and for the 1.1 mg K^+ nanoparticles, it was from 38.2 min to 37.5 min (+0.2 mA) and 38.8 min (–0.2 mA). On day 56, significant differences in RT were recorded, with a longer RT ranging from 25.9–0.3 min and 37.3–38.7 min for P1 and P2, respectively (**Figure 4.10b** and **Table 4.3**).

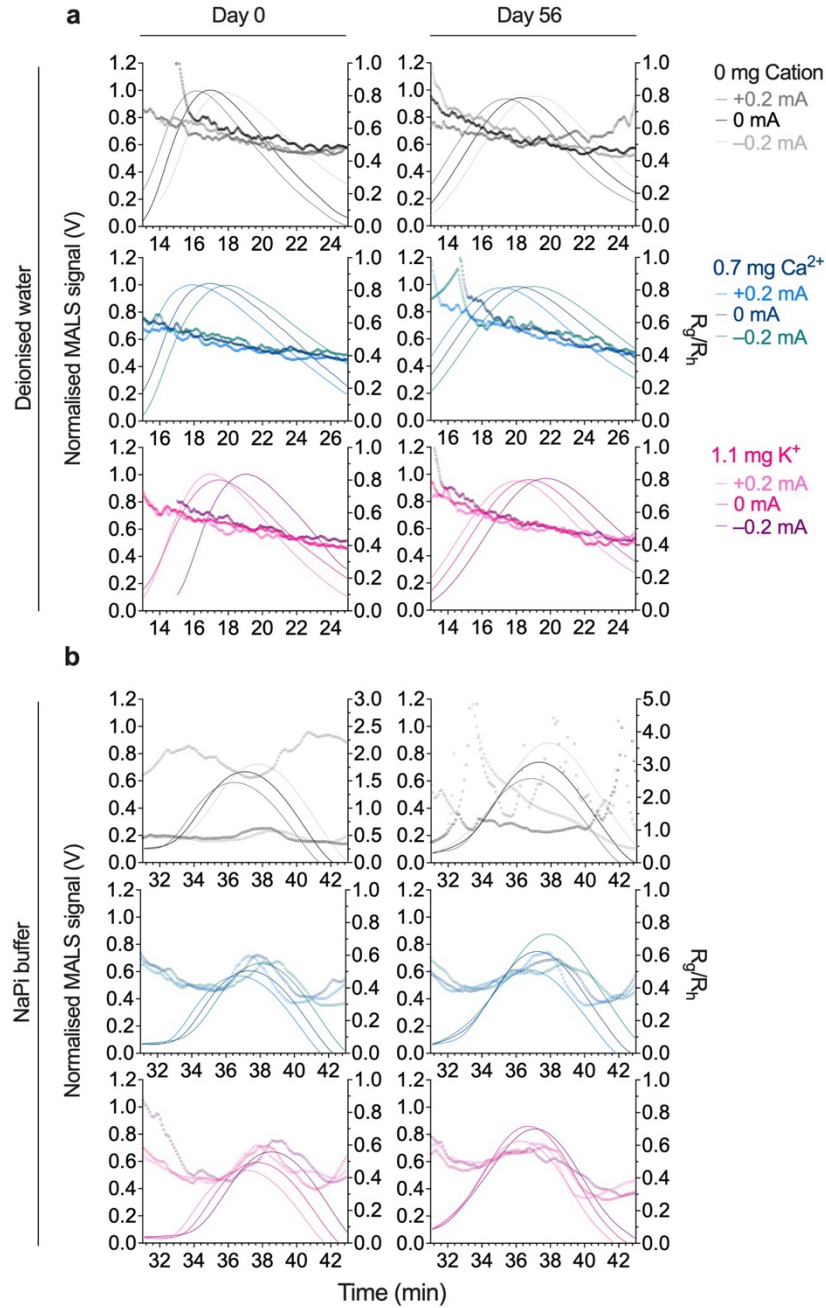


Figure 4.10 Electrical asymmetric flow field-flow fractionation (EAF4) fractograms of silk nanoparticles dispersed in deionized water and 10 mM sodium phosphate (NaPi) buffer (pH 7.4). Silk nanoparticles (0.1 mg/mL) were separated in 0.5 mM sodium carbonate under electrical fields with applied positive (+0.2 mA), neutral (0 mA), and negative (−0.2 mA) currents. Nanoparticles EAF4 fractograms for day 0 and day 56 storage were presented as a function of normalized MALS signal and shape factor (R_g/R_h): (a) deionized water storage and (b) NaPi buffer (pH 7.4) storage. The experiment was conducted as three technical repeats each ($n = 3$).

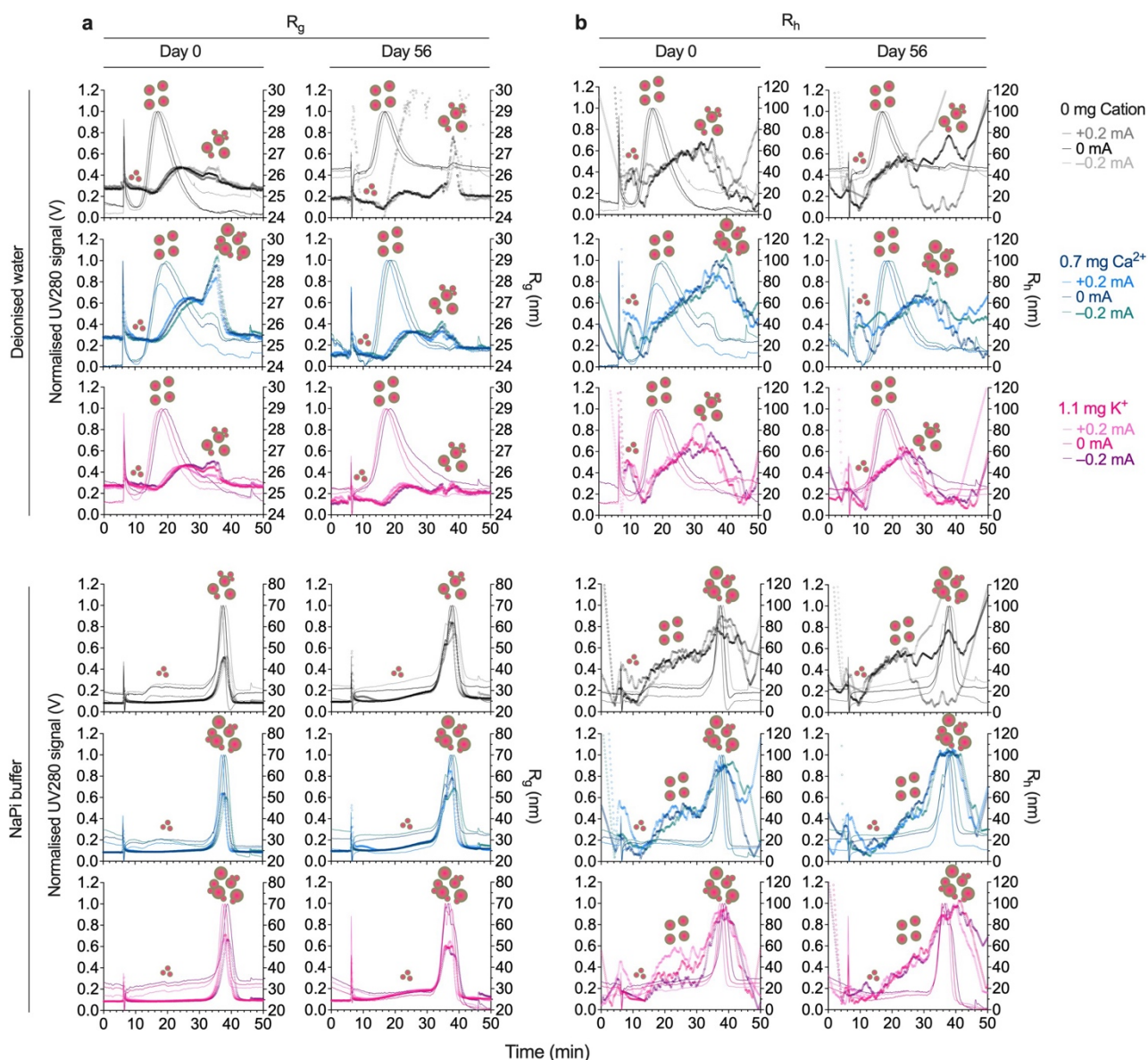


Figure 4.11 Electrical asymmetric flow field-flow fractionation (EAF4) fractograms of silk nanoparticles (SNPs): 0 mg Cation SNP (black), 0.7 mg Ca^{2+} SNP (blue), and 1.1 mg K^+ SNP (pink). SNPs (0.1 mg/mL) were separated using a 0.5 mM sodium carbonate carrier liquid under with applied positive (+0.2 mA), neutral (0 mA), and negative (−0.2 mA) currents. The SNP EAF4 fractograms for days 0 and 56 of storage in deionized water and 10 mM Sodium phosphate (NaPi) buffer (pH 7.4) at 4 °C presented as a correlation of normalized UV 280 signal together with (a) radius of gyration (R_g) and (b) hydrodynamic radius (R_h). The experiment was conducted as three technical repeats each ($n = 3$).

The R_g and R_h for P1 identified under deionized water conditions showed R_g and R_h in the ranges of 27.8–29.5 nm and 31.4–65.9 nm, respectively. These values were significantly different from the values on day 0. The R_g and (R_h) for P2 were higher than for the majority of particles dispersed in deionized water, with particle sizes of 45.5–46.1 nm (73.4–79.1 nm), 50.0–51.9 nm (81.8–94.5 nm), and 52.3–57.7 nm (85.3–93.0 nm) for 0 mg Cation, 0.7 mg Ca^{2+} , and 1.1 mg K^+ nanoparticles, respectively. The R_g after 56 days was similar; however, no significant changes in R_h were detected for the 0.7 mg Ca^{2+} and 1.1 mg K^+ nanoparticles after 56 days of storage, whereas the 0 mg Cation nanoparticles showed a wide range of R_h values from 8–136.7 nm. The shape factor of the 0 mg Cation nanoparticles was clearly influenced, as the nanoparticles displayed an increased and fluctuating shape factor value from day 0 (0.57–0.64) to day 56 (0.94–18.3). The shape retention of the 0.7 mg Ca^{2+} and 1.1 mg K^+ nanoparticles was greater, with shape factors of approximately 0.55–0.64 and 0.45–0.61 for day 0 and day 56, respectively. Smaller-sized particles were indicated, as P1 showed no significant changes in shape factor, ranging from 0.50 to 0.70, during storage (**Figure 4.8a-b** and **Table 4.3**).

Assessment of the recovery percentage of nanoparticles dispersed in sodium phosphate buffer (pH 7.4) revealed recovery rates of $70 \pm 6\%$ for 0 mg Cation, $82 \pm 6\%$ for 0.7 mg Ca^{2+} , and $92 \pm 7\%$ for 1.1 mg K^+ nanoparticles, however; the rate was slightly changed after 56 days, at $74 \pm 9\%$, $87 \pm 3\%$, and $87 \pm 5\%$, respectively. The zeta potential could not be calculated here because the main peak was eluted without applying a cross flow. Silk nanoparticle fractograms detected with the UV 280 detector were conducted under ± 0.2 mA currents and are shown along with the particle size data (R_g and R_h) in the supplementary section (**Data not shown**).

Table 4.2 Parameters measured for silk nanoparticles dispersed in deionized water derived from electrical asymmetric flow field-flow fractionation (EAF4)-MALS. Two-way ANOVA and Šidák's multiple comparisons test were used in statistical analysis, comparing day 56 and 0 data; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Abbreviations: Peak 1 (P1); Peak 2 (P2); hydrodynamic radius (R_h); radius of gyration (R_g); dynamic light scattering (DLS); zeta potential (ZP).

Silk nanoparticle	Applied current (mA)	Elution time (min)		MALS-R _g (nm)		DLS-R _h (nm)		R _g /R _h		Electrophoretic mobility (mm cm/V s)	Correlation coefficient (R ²)	ZP (mV)
		P1	P2	P1	P2	P1	P2	P1	P2			
Day 0												
0 mg Cation	+0.2	17.3 (±0.0)	32.8 (±0.5)	25.5 (±0.1)	25.9 (±0.0)	38.2 (±1.6)	53.8 (±15.8)	0.67 (±0.03)	0.51 (±0.14)	-4.71	0.9841	-60.30
	0	18.1 (±0.1)	32.7 (±0.2)	25.5 (±0.1)	26.1 (±0.0)	36.7 (±1.8)	67.1 (±19.9)	0.70 (±0.04)	0.42 (±0.41)			
	-0.2	18.9 (±0.1)	32.5 (±0.1)	25.6 (±0.1)	26.2 (±0.1)	39.7 (±5.3)	62.9 (±1.9)	0.65 (±0.09)	0.42 (±0.01)			
0.7 mg Ca2+	+0.2	20.1 (±0.4)	32.7 (±0.2)	26.2 (±0.1)	27.5 (±0.2)	44.2 (±2.0)	85.0 (±5.8)	0.59 (±0.03)	0.33 (±0.03)	-4.78	0.9864	-61.24
	0	21.0 (±0.1)	33.4 (±0.4)	26.1 (±0.0)	28.0 (±0.2)	46.5 (±1.0)	78.1 (±10.6)	0.56 (±0.01)	0.36 (±0.05)			
	-0.2	22.0 (±0.2)	33.3 (±0.1)	26.0 (±0.1)	28.0 (±0.1)	46.4 (±3.4)	71.4 (±9.8)	0.56 (±0.04)	0.40 (±0.06)			
1.1 mg K+	+0.2	18.0 (±0.1)	32.5 (±0.1)	25.5 (±0.1)	25.9 (±0.1)	42.5 (±1.7)	85.8 (±7.2)	0.60 (±0.02)	0.30 (±0.03)	-4.78	0.9795	-61.15
	0	19.1 (±0.1)	32.7 (±0.3)	25.6 (±0.1)	26.1 (±0.1)	43.3 (±1.0)	63.9 (±6.9)	0.70 (±0.01)	0.41 (±0.05)			
	-0.2	20.1 (±0.1)	32.8 (±0.1)	25.6 (±0.1)	26.4 (±0.1)	42.6 (±4.7)	64.6 (±15.3)	0.61 (±0.07)	0.42 (±0.10)			
Day 56												
0 mg Cation	+0.2	16.8 (±0.2)*	37.7 (±0.1)****	24.0 (±1.2)****	28.1 (±0.2)*	42.2 (±0.9)	5.5 (±0.7)****	0.57 (±0.04)*	5.12 (±0.56)****	-4.84	0.9734	-62.02
	0	17.7 (±0.1)	38.4 (±0.1)****	24.7 (±0.1)*	27.8 (±0.1)	44.8 (±0.5)**	37.9 (±21.3)*	0.55 (±0.01)***	0.88 (±0.40)			
	-0.2	18.6 (±0.2)	39.0 (±0.1)****	24.7 (±0.1)**	23.6 (±5.0)**	43.1 (±0.7)	101.4 (±23.1)***	0.57 (±0.01)	0.24 (±0.11)			
0.7 mg Ca2+	+0.2	18.0 (±0.2)****	33.7 (±0.2)****	24.8 (±0.1)****	25.6 (±0.1)	49.0 (±2.2)	25.9 (±6.4)****	0.51 (±0.02)	1.03 (±0.29)	-4.22	0.9873	-54.07
	0	19.6 (±0.1)****	33.3 (±0.1)	24.8 (±0.1)****	25.6 (±0.1)*	49.0 (±2.0)	29.2 (±2.7)****	0.51 (±0.02)	0.88 (±0.08)			
	-0.2	20.7(±0.2)****	33.7 (±0.2)	24.9 (±0.0)****	25.9 (±0.1)*	49.4 (±1.7)	40.4 (±5.8)**	0.50 (±0.02)	0.66 (±0.09)			
1.1 mg K+	+0.2	17.6 (±0.2)*	32.8 (±0.2)	24.8 (±0.1)*	25.1 (±0.1)	44.2 (±1.4)	4.0 (±2.3)****	0.56 (±0.02)	5.52 (±4.29)****	-3.74	0.8712	-47.95
	0	18.3 (±0.4)****	32.9 (±0.2)	24.9 (±0.1)*	25.0 (±0.1)	44.9 (±1.0)	8.4 (±1.1)****	0.55 (±0.01)	3.02 (±0.40)*			
	-0.2	19.3 (±0.1)****	33.3 (±0.1)*	24.8 (±0.0)**	25.2 (±0.0)	45.3 (±1.1)	13.4 (±6.3)****	0.59 (±0.01)	2.73 (±1.33)			

Table 4.3 Parameters measured for silk nanoparticles dispersed in 10 mM sodium phosphate buffer (pH 7.4) derived from electrical asymmetric flow field-flow fractionation (EAF4)-MALS. Two-way ANOVA and Šídák's multiple comparisons test were used, comparing day 56 and 0 data: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Abbreviations: Peak 1 (P1); Peak 2 (P2); hydrodynamic radius (R_h); radius of gyration (R_g); dynamic light scattering (DLS); zeta potential (ZP); no data available (NA).

Silk nanoparticle	Applied current (mA)	Elution time (min)		MALS-R _g (nm)		DLS-R _h (nm)		R _g /R _h	
		P1	P2	P1	P2	P1	P2	P1	P2
Day 0									
0 mg Cation	+0.2	17.7 (±0.4)	37.0 (±0.1)	24.6 (±0.1)	46.1 (±0.1)	33.7 (±2.6)	73.4 (±13.9)	0.73 (±0.06)	0.64 (±0.14)
	0	18.9 (±0.3)	37.7 (±0.1)	24.5 (±0.1)	45.5 (±1.0)	35.9 (±9.8)	79.1 (±3.3)	0.73 (±0.23)	0.57 (±0.01)
	-0.2	20.2 (±0.9)	38.4 (±0.1)	24.4 (±0.1)	45.8 (±0.6)	31.6 (±4.3)	76.4 (±16.4)	0.78 (±0.10)	0.62 (±0.14)
0.7 mg Ca ²⁺	+0.2	21.3 (±0.2)	37.3 (±0.1)	24.7 (±0.1)	51.7 (±0.3)	38.0 (±5.3)	94.5 (±5.3)	0.66 (±0.10)	0.64 (±0.11)
	0	22.1 (±0.1)	37.9 (±0.1)	24.6 (±0.1)	51.9 (±0.3)	33.5 (±9.1)	81.8 (±12.8)	0.77 (±0.18)	0.55 (±0.03)
	-0.2	23.3(±0.3)	38.5 (±0.0)	24.6 (±0.1)	50.0 (±0.3)	31.6 (±3.7)	87.5 (±4.5)	0.79 (±0.09)	0.57 (±0.03)
1.1 mg K ⁺	+0.2	20.4 (±0.4)	37.5 (±0.0)	24.7 (±0.1)	57.7 (±0.6)	50.4 (±9.2)	89.2 (±6.3)	0.50 (±0.10)	0.65 (±0.04)
	0	21.5 (±0.5)	38.2 (±0.0)	24.4 (±0.1)	55.5 (±0.4)	31.6 (±4.1)	93.0 (±0.7)	0.78 (±0.10)	0.60 (±0.01)
	-0.2	22.7 (±0.3)	38.8 (±0.1)	24.3 (±0.1)	53.2 (±0.6)	25.3 (±2.4)	85.3 (±5.1)	0.97 (±0.10)	0.63 (±0.03)
Day 56									
0 mg Cation	+0.2	25.9 (±0.1)****	37.5 (±0.1)****	27.9 (±0.3)****	62.2 (±1.3)****	31.4 (±14.0)	8.0 (±5.8)****	0.99 (±0.35)	18.30 (±21.95)**
	0	30.3 (±0.3)****	38.1 (±0.1)****	29.5 (±0.3)****	61.8 (±0.3)****	56.5 (±11.7)	78.3 (±42.6)	0.54 (±0.12)	0.94 (±0.44)
	-0.2	29.8 (±0.8)****	38.7 (±0.1)****	29.3 (±0.3)****	56.7 (±0.3)****	65.9 (±34.4)*	136.7 (±108.8)****	0.57 (±0.37)	1.38 (±1.88)
0.7 mg Ca ²⁺	+0.2	26.3 (±0.5)****	37.5 (±0.1)*	27.9 (±0.3)****	62.5 (±1.4)****	45.0 (±6.1)	102.7 (±2.3)	0.63 (±0.08)	0.61 (±0.02)
	0	26.6 (±0.0)****	38.0 (±0.1)	28.0 (±0.2)****	59.1 (±0.6)****	48.3 (±19.9)	103.5 (±10.2)	0.64 (±0.21)	0.57 (±0.05)
	-0.2	26.6 (±0.0)****	38.7 (±0.0)**	27.8 (±0.1)****	54.3 (±0.4)****	39.7 (±9.9)	99.9 (±5.7)	0.73 (±0.17)	0.54 (±0.03)
1.1 mg K ⁺	+0.2	28.4 (±0.3)****	37.3 (±0.1)*	29.5 (±0.2)****	52.2 (±0.5)****	42.2 (±4.1)	102.7 (±2.3)	0.70 (±0.07)	0.51 (±0.01)
	0	29.0 (±0.4)****	37.8 (±0.0)****	29.1 (±0.1)****	50.0 (±0.3)****	52.9 (±19.1)	103.7 (±9.0)	0.61 (±0.25)	0.48 (±0.04)
	-0.2	29.6 (±0.6)****	38.2 (±0.1)****	29.0 (±0.2)****	46.1 (±0.2)	45.5 (±7.4)	102.1 (±5.1)	0.65 (±0.11)	0.45 (±0.02)

4.4. Discussion

4.4.1. The Impact of Counterions on Silk Nanoparticles Colloidal Stability

The diffusion self-interaction parameter, k_D , can be used to estimate particle interaction behavior in a particular dispersant as a predictor of physicochemical stability. A negative k_D value indicates a slow diffusion rate due to aggregation or clustering (attractive force), while a positive k_D value indicates a higher diffusion rate, which causes a greater dispersity of particles (repulsive force).^{280, 281} Therefore, the increased particle size with a less negative k_D produced in PBS and normal saline indicated silk nanoparticle aggregation (**Table 4.1**). A possible explanation for these observations is a salting-out effect at higher ionic strength—resulting from the presence of monovalent and divalent cations, including Ca^{2+} and K^+ , as well as phosphate salts in the buffer. I hypothesized that these components influence surface charge shielding and modulate the electrical double layer,^{282, 283} thereby reducing electrostatic repulsion between particles, which leads to decreased solubility and increased aggregation.^{284, 285} This work also indicates that the instability of silk nanoparticles in PBS and normal saline should be further evaluated, as well as the effects of the biomolecular corona, as this may enhance our understanding of their behaviour in biologically relevant media for biological testing.

4.4.2. The Impact of Counterions on Physicochemical Characteristics of Silk Nanoparticles in Deionized Water and Sodium Phosphate Buffer

The ionic strength and pH of the continuous phase both play an important role in colloidal stability, impacting the protonation and deprotonation state of charged solvent-exposed amino acid residues, silk protein structures, and conformational stability.²⁸⁴⁻²⁸⁶ Therefore, I also examined silk nanoparticle colloidal stability at pH 5.8–8.0 in sodium phosphate buffer (**Figure 4.2b** and **Table 4.1**). The pH of the extracellular (pH 6–6.5) and endo-lysosomal (pH 4.5)

microenvironments of cancer cells¹⁸⁰ which could influence the performance and effectiveness of nanocarriers. Reduced particle stability (e.g., agglomeration) under these pH conditions could adversely impact both cellular and intracellular delivery,^{287, 288} increase the risk of cytotoxicity to normal cells,²⁶⁴ and delay the release of encapsulated drug cargoes.²⁶⁴

These observations highlight key factors involved in silk nanoparticle agglomeration such as surface charge and particle concentration. At a constant protein concentration, the number of smaller nanoparticles was higher than that of larger nanoparticles (**Figure 4.3a**). This increase in particle number led to greater particle interactions and collisions,^{289, 290} promoting agglomerate growth. Therefore, the smaller-sized particles exhibited a higher aggregation rate.

The theoretical isoelectric point (pI) for silk H-chain is 4.39;³³ thus, low pH conditions would induce less deprotonation, resulting in a lower net negative charge. This trend has been reported elsewhere, demonstrating that acidic conditions can modulate aggregate size and increase the volume of silk protein.^{291, 292} Furthermore, silk nanoparticles exhibited a strong negative zeta potential of approximately -45 mV. I propose that an increased protonated state of the NH_2 group and/or destabilization of the counter ions ($\text{Ca}^{2+}/\text{K}^+$) could explain this observation, as this would lead to a reduced particle net surface charge (less negative) and alter the protein conformation/particle shape, resulting in reduced interparticle repulsive interactions to drive particle agglomeration.²⁸⁶ However, silk nanoparticles with the counter ions ($\text{Ca}^{2+}/\text{K}^+$) ameliorate particle agglomeration comparable to control silk nanoparticle (0 mg cation).

Silk nanoparticles act like a charge-shifting nanoparticle, offering a promising strategy for designing pH-responsive silk nanoparticles for targeted drug delivery with tunable cellular uptake (100–200 nm diameter)²⁹³ and controlled release properties.²⁹⁴ Proof of principle comes from control silk nanoparticles that showed pH responsiveness drug release,²⁹⁵ charge dependent silk-drug interactions,²⁹⁶ and lysosomotropic drug delivery in single live cells.²⁹⁷

Given that silk nanoparticles in deionized water and sodium phosphate buffer exhibited no significant signs of agglomeration during short-term stability studies (2 hours), the long-term storage stability of nanoparticles in these media was investigated for up to 56 days at 4 °C. Silk nanoparticles dispersed in deionized water had excellent stability, with no changes in physicochemical properties observed (i.e., particle size, concentration, PDI, and charge) relative to day 0. In contrast, a small change in these parameters was observed in sodium phosphate buffer, with increased particle size, reduced particle concentration, and changes in PDI value. However, no changes in zeta potential were detected under these conditions.

Loss of colloidal stability was observed from day 7, particularly in the case of smaller-sized silk nanoparticles (82.7–97.3 d.nm). One possible explanation for this observation is the occurrence of ionic strength-induced particle aggregation achieved by reducing electrostatic repulsion between particles and promoting cluster formation with a broader size distribution (**Figure 4.3a** and **Figure 4.4a**). These results align with the FE-SEM micrographs captured on day 56, which illustrated the presence of greater numbers of aggregated silk nanoparticles in comparison to day 0. Conversely, no significant changes were detected for nanoparticles stored in deionized water (**Figure 4.3b** and **Figure 4.4b**). I speculate that, in this case, the particle size (~100 d.nm) and concentration ($\geq 2 \times 10^{11}$ particle/mL) may play a vital role in regulating the silk nanoparticle colloidal stability^{289, 290} in sodium phosphate buffer at physiological pH, rather than the particle surface net charge. In the absence of an added counterion ($\text{Ca}^{2+}/\text{K}^{+}$), and at 37 °C, the 0 mg cation silk nanoparticles exhibited reduced physicochemical stability at day 7, appearing as micron-sized agglomerates (~5 μm) with higher polydispersity and a lower net charge compared to the smaller-sized nanoparticles formulated at lower Ca^{2+} (0.7 mg) and K^{+} (1.1 mg) concentrations (**Figure 4.5**). These results indicated that Ca^{2+} and K^{+} may promote nanoparticle stability at higher storage temperatures by sustaining a repulsive hydration force

on the particle surface,²⁹⁸ stabilizing the silk nanoparticle core structure via β -sheet content,⁹⁷ and preventing particle agglomeration. The thioflavin T assay confirmed the improved hydrophobic core strength in the presence of counterions, as the thioflavin T molecule can recognize β -sheet structures (reviewed in²⁵²). Ca^{2+} and K^{+} significantly increase thioflavin T entrapment into particles, while antisolvent precipitation occurs (**Figure 4.7**).

Taken together, these findings indicate that Ca^{2+} showed greater promise than K^{+} for increasing the silk nanoparticle physical properties and gave rise to nanoparticles with higher stability under acidic conditions and elevated temperature, and that showed less alteration in agglomeration behavior (*via* the DLS, NTA, and FE-SEM techniques) and net surface charge (*via* the ELS technique). One possible explanation is that counterions modulate silk conformation during nanoparticle formation through the formations of: (1) transient salt bridges with negatively charged residues located at the hydrophilic spacer and N-terminus of silk H-chain,²⁸ which could be presented on the nanoparticle surface, and (2) coordination complexes with other amino acid residues in hydrophobic region such as histidine,²⁸ serine,¹⁴ and tyrosine,^{15, 110} addressed as an organizing and stabilizing β -sheet formation (reviewed in¹⁹²), which could be presented inside the nanoparticle core. Due to the differences in ionic radii between Ca^{2+} (0.99 Å) and K^{+} (1.33 Å),³⁶ the Ca^{2+} may exhibit a stronger bonding with carbonyl oxygen, thereby constructing a more appropriate network of π - π interacting tyrosines^{15, 110} and a stronger interaction with negatively charged residues.²⁶⁶ Therefore, I propose that these factors may give the Ca^{2+} nanoparticles a higher particle physical strength. An increased temperature drives protein denaturation/unfolding by disrupting hydrogen bonds, electrostatic interactions, and hydrophobic forces,^{299, 300} as well as a denaturation caused by acidic pH (pH 5.8) (described above). This may initiate a leakage of cations from less intact K^{+} silk nanoparticles, especially those of larger particle size (17.3 mg K^{+} nanoparticles), resulting

in reduced zeta potential and increased agglomeration (**Figure 4.2b**, **Table 4.1**, and **Figure 4.5**).

Although I observed that counterion inclusion in silk nanoparticle formulations minimized agglomeration behavior for up to 56 days under refrigerated storage conditions, the resulting increase in particle size remained within acceptable specifications for nanomedicine-based applications (**Figure 4.3** and **Figure 4.4**). Nanoparticles in the 100–500 nm size range contribute to passive accumulation in tumor masses *via* the enhanced permeability and retention (EPR) effect,²⁵⁸ while particles with 600 nm in size can provide penetration ability to hair follicles, which is beneficial for dermal drug delivery.²⁶⁰ Therefore size tuning offers the potential to unlock a range of therapeutic applications beyond cancer.

4.4.3. Silk Nanoparticle Physical Stability Studied with Asymmetric Flow Field-Flow Fractionation with Online UV-Multiangle Light Scattering, and Dynamic Light Scattering (AF4-UV-MALS-DLS)

The AF4 analysis provided a more comprehensive insight for interparticle interactions and particle structure, indicating a minor yield of aggregates during a separation analysis in 0.5 mM Na₂CO₃ (pH ~10) for the 0.7 mg Ca²⁺ silk nanoparticles rather than for the control (0 mg cation) or 1.1 mg K⁺ silk nanoparticles. The same result was not detectable using DLS or NTA measurements; however, it was consistent with a colloidal stability test in the basic condition, with dispersal in sodium phosphate buffer (pH 8.0), where the increased pH led to a high chance of aggregation (more negative k_D) of the 0.7 mg Ca²⁺ nanoparticles, whereas the other silk nanoparticles showed an opposite trend (less negative k_D) (**Table 4.1**). In addition, with a noticeable increase in particle size in the 0.7 mg Ca²⁺ nanoparticles (**Figure 4.9a**). I speculate that a high pH (pH 8–10) reduced the stability of the 0.7 mg Ca²⁺ nanoparticles to a greater extent than for the other silk nanoparticles through several possible pathways, such as protein denaturation, particle integrity disruption, and net charge surface alteration.²⁸⁴⁻²⁸⁶

The AF4-UV-MALS-DLS provided us with more insights into particle size and morphological properties. As the particle diffuses/moves through the solution, the radius of gyration (R_g), measured by the inline MALS detector, along with a hydrodynamic radius (R_h) measured by the inline DLS, can be determined. The hydrodynamic radius (R_h) refers to the radius of a hard sphere that diffuses in the same way as the particle, so it includes the actual particle and the solvation shell. Therefore, a larger particle will experience a larger friction moving in solution and thus have a larger R_h .^{301, 302} The radius of gyration (R_g) refers to the distribution of mass around the center of the particle mass, indicating a particle structure, as a compact particle will have a small R_g and an extended/loosened particle will have a larger R_g .³⁰³ The ratio of R_g to R_h , referred to as the shape factor, is commonly used to investigate the shape and retention-related properties of particles in solution. A spherical shape possesses an R_g/R_h of ~ 0.755 .³⁰¹

The AF4 investigation study initially revealed a considerable impact of counterion inclusion (Ca^{2+} and K^+) on the retention, size, and shape of silk nanoparticles during separation in a flowing system. According to the separation profiles of the control (0 mg cation) and 1.1 mg K^+ silk nanoparticles, particles with a more spherical shape and a tightly packed structure could potentially be separated from the crude silk particle population. These particles eluted as the major peak (P1), confirming their small R_g and R_h values, with shape factors close to the ideal value of 0.755.³⁰¹ This indicated a high colloidal stability of nanoparticles even when exposed to high pH conditions for 1 h. However, the 0.7 mg Ca^{2+} nanoparticles exhibited noticeable colloidal instability (increased aggregation), together with a shape distortion that deviated from the ideal spherical shape (from 0.755¹⁹² to 0.56), and represented most particles eluting as P1. The evidence from this study suggests that Ca^{2+} incorporation into silk nanoparticles improved the structural stability and particle-solvent interaction to a greater extent than was possible with the control (0 mg cation) or 1.1 mg K^+ silk nanoparticles at a

basic condition (pH ~10). Loosening the particle structure (larger R_g) and increasing the solvation layer (larger R_h) led to a longer retention time (i.e., longer traveling time in solution due to low diffusivity and more friction) (**Figure 4.3c**, **Figure 4.10a**, and **Table 4.2**).

Comparison of the valence state and ionic radii between Ca^{2+} and K^+ reveals that Ca^+ is more capable than K^+ of forming strong charge-charge or electrostatic interactions with proteins.^{15, 36, 110, 266} However, the charged residues on proteins under AF4 separation and the changes incurred by an increase in pH (silk becomes more negative)³³ may disrupt the ordered silk conformation and/or silk-counterion interactions, thus reducing the particle integrity, distorting the particle shape, and promoting particle aggregation. However, after 56 days of storage, the 0.7 mg Ca^{2+} silk nanoparticles showed fewer aggregates compared to day 0. The alterations may have only a minor effect, leading to reversible, transient aggregates at day 56.

Interestingly, the shape-retention stability of the silk nanoparticles dispersed in deionized water, as demonstrated by the FE-SEM micrographs, showed fewer aggregates and smooth, round particles up to day 56 (**Figure 4.3b**). Therefore, the inconsistency between the computed shape factor and the FE-SEM micrograph, especially for the 0.7 mg Ca^{2+} nanoparticles, may be due to an influence of the carrier liquid composition and pH during the AF4 separation.

The silk nanoparticle fractograms studied with the UV 280 nm signal were also reported and showed a correlation between the UV signal and the particle size data for both R_g and R_h (**Figure 4.11**). However, the particle size for R_g (R_h in parentheses) in the range of 25.5–26.1 nm (36.7–43.4 nm) accounted for the majority of nanoparticles dispersed in deionized water, indicating a narrow size distribution of uniform and nonaggregated particles. In contrast, an increased particle size was observed in sodium phosphate buffer (pH 7.4), with both R_g and R_h increasing approximately 2-fold for each sample. I hypothesized that the physicochemical attributes of the silk nanoparticles dispersed in sodium phosphate buffer (pH 7.4) had

undergone a slight change based on the alteration in diffusivity shown in **Figure 4.2a** (increased R_h), as well as a loss in particle integrity (increased R_g) and an increase in particle agglomeration, as shown in **Figure 4.4b** (increased R_g and R_h). Interestingly, the shape factor was still maintained in deionized water (ranging from 0.55–0.65), suggesting a slight deviation from a spherical shape (**Figure 4.10b** and **Table 4.3**). Storage for 56 days significantly altered the physicochemical stability of the 0 mg Cation nanoparticles to a greater extent than was observed for the 0.7 mg Ca^{2+} and 1.1 mg K^+ silk nanoparticles stored in sodium phosphate buffer (pH 7.4) buffer. The 0 mg Cation nanoparticles changed significantly in size (R_g and R_h) and shape (>1) (**Figure 4.10b** and **Table 4.3**). A potential reason for this was the lower colloidal stability of the 0 mg Cation nanoparticles (with a lower k_D of -2.373) (**Table 4.1**). In the absence of counterions (Ca^{2+} and K^+), the repulsive interactions between particles and the particle–solvent interactions were weakened and/or unsteady, leading to enhanced agglomeration, fluctuating R_g and R_h values, and an extended shape. Conversely, the silk nanoparticles with 0.7 mg Ca^{2+} and 1.1 mg K^+ , when stored in sodium phosphate buffer (pH 8.0), exhibited excellent physical stability in terms of both size and shape retention, as reflected in their flow behaviors, packing densities, and surface areas. Due to limited measurement repeats, the FE-SEM dataset was obtained from a single experimental run. Although the observed trends were supported by DLS, NTA, AF4, and EAF4 analyses, the FE-SEM results should be interpreted with caution regarding statistical reliability. To enable particle charge analysis via the EAF4 separation system, an alkaline carrier solution (Na_2CO_3 , pH ~ 10) was used. However, this condition may not fully represent a biologically relevant environment; therefore, these datasets were interpreted within the context of the experimental system only. Despite this limitation, the findings provide useful insight into silk nanoparticle behavior and may support the design and optimization of nanoparticles with tailored physicochemical properties. Future studies should further explore the use of different carrier liquids.

4.4.4. Silk Nanoparticle Surface Charges Characteristic Studied with Electrical Asymmetric Flow Field-Flow Fractionation (EAF4-UV-MALS-DLS)

The EAF4 fractograms displayed an overlay of a normalized MALS signal together with the shape factor (R_g/R_h). The silk nanoparticles separated based on their size (diffusion) and charge (zeta potential) properties under an electric field created by applying positive (+0.2 mA) and negative (−0.2 mA) currents. The nanoparticles exhibited a slight shift of the first peak (P1) to a lower elution time when applying a positive current and a shift in the opposite direction toward a higher RT when applying a negative current.

Shape factor analysis using the EAF4 system indicated that nanoparticles had a predominantly spherical morphology, as a homogenous hard sphere has a shape factor ~ 0.755 ³⁰¹ (**Figure 4.10a** and **Table 4.2**). Larger aggregates exhibited a lower shape factor value, consistent with the FE-SEM results (**Figure 4.3b**). Interestingly, after 56 days of storage in deionised water, the positive current altered the particle morphology, increasing the shape factor to over 0.755, indicating a hollow sphere and/or a rod shape (**Figure 4.10a** and **Table 4.2**).^{301, 302}

EAF4, which combines AF4 and electrical field-flow fractionation, provides charge- and size-dependent separation of analytes.^{141, 304} Therefore, using EAF4, nanoparticle characteristics can be further probed in response to the application of an electric field, with nanoparticles expected to exhibit significant responses to an applied electrophoretic force under a ± 0.2 mA current to the top electrode, which would shift the main elution peak (P1) relative to neutral conditions (absence of applied current, 0 mA) (**Figure 4.10**). These findings suggest that the positive top electrode (+0.2 mA) accelerates the movement of silk nanoparticles toward the membrane according to the particle size and charge density while simultaneously increasing the negative surface charge of the membrane. This shortens the retention time due to increased electrostatic repulsion effects between silk nanoparticles and the membrane.¹⁴⁹ Conversely,

applying a negative current to the top electrode (-0.2 mA) alters the nanoparticle–membrane interaction and increases the electrostatic attractive forces generated by the bottom electrode, resulting in a longer retention time.¹⁴⁹ Interestingly, electrophoretic force appeared to have the most significant impact on 0.7 mg Ca^{2+} nanoparticles, resulting in significant separation profiles by decreasing the proportion of nanoparticles under an applied positive current and promoting agglomeration under an applied negative current.

The zeta potential of the 0.7 mg Ca^{2+} nanoparticles was less negative than that of the control silk nanoparticles (0 mg cation) and 1.1 mg K^{+} nanoparticles. These properties support the assumption that an applied positive current of 0.2 mA may induce aggregation and adsorption of 0.7 mg Ca^{2+} nanoparticles relative to the neutral and applied negative current conditions. These data were aligned with the particle characteristics of 0.7 mg Ca^{2+} nanoparticles studied with AF4 and revealed the formation of a transient nanoparticle complex. However, I suggest that this represents a minor issue for silk nanoparticle formulation and characterization, as it resulted in a high recovery percentage of $>80\%$.

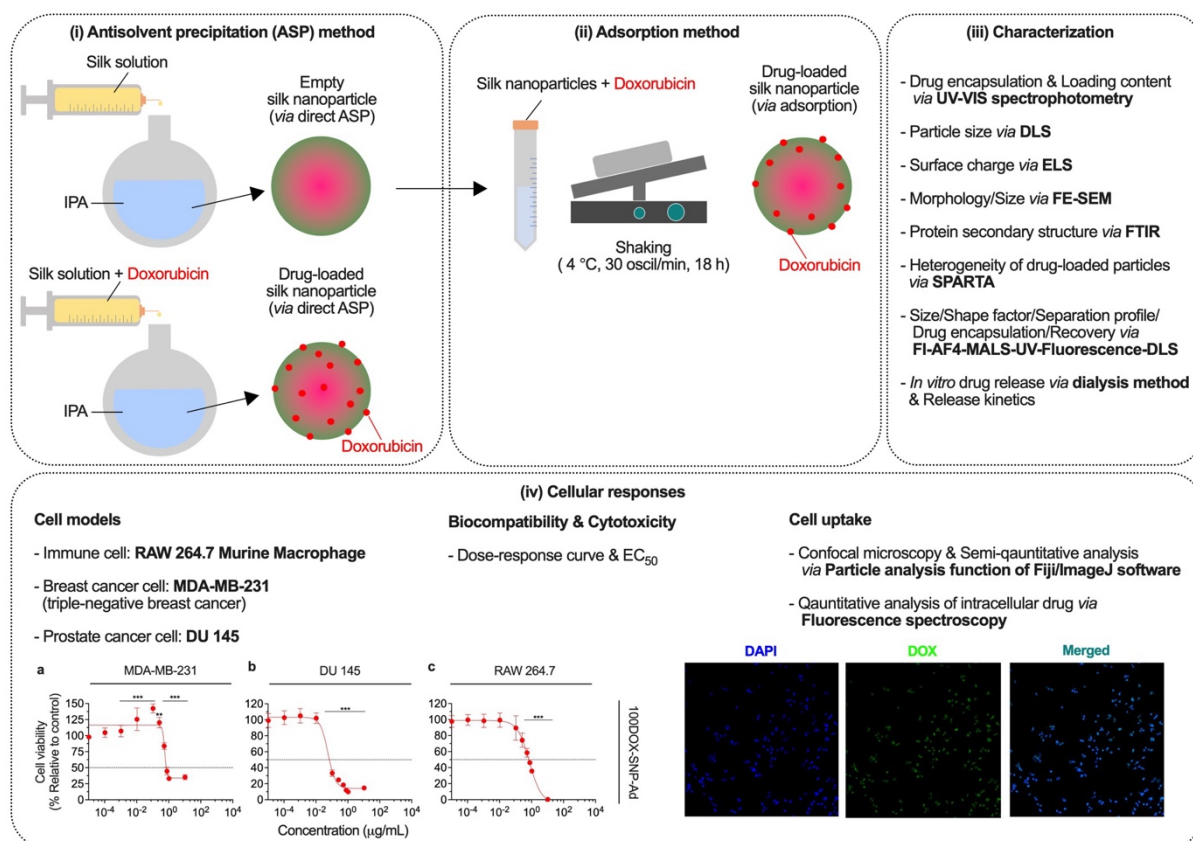
The EAF4 data confirmed the role of counterions (Ca^{2+} and K^{+}) in stabilizing the colloidal stability of silk nanoparticles during storage in both deionized water and sodium phosphate buffer (pH 7.4). When the nanoparticles were observed under the electric field, the EAF4 analysis revealed the particle properties in response to charge and strengthened the AF4 analysis by showing that the counterions play a crucial role in improving particle interactions through their charge properties (resulting in fewer/transient aggregates). This also helps to maintain the particle structure and shape of the majority of the silk nanoparticles (shape factor: 0.50 – 0.59 for deionized water and 0.48 – 0.61 for sodium phosphate buffer at pH 7.4). In contrast, a noticeable distortion in particle shape and size was routinely observed for the control silk nanoparticles (0 mg Cation) (**Figure 4.10**, **Table 4.2**, and **Table 4.3**)

Taken together, these EAF4 findings indicated favorable properties for silk nanoparticles separated by particle size and charge. I propose that these findings can initially reveal the interparticle interactions occurring in a flowing system as well as the particle–membrane interaction, thereby reflecting the promising biochemical functions of negatively charged nanoparticles in clinical situations, such as intratumoral distribution enhancement (reviewed in³⁰⁵) and skin penetration enhancement (reviewed in³⁰⁶). Due to the limited range of carrier liquids that can be used for EAF4 applications, further investigations into carrier liquid composition and its impact on EAF4 analyses are required to charter this underexplored area of research

4.5. Conclusion

Orthogonal analytical methods were used to investigate the physical stability of silk nanoparticles formulated with cations (Ca^{2+} and K^{+}). All nanoparticles exhibited acceptable short-term physical stability when dispersed in deionized water and phosphate buffer. Further profiling of the long-term storage stability of these nanoparticles revealed negligible changes in particle size and charge following storage in deionized water, but significant changes were observed by day 7 following storage in sodium phosphate buffer (pH 7.4). This is the first use of conventional and electric asymmetric flow field flow fraction modalities with inline light scattering to probe changes in nanoparticle size, charge, and shape. I presented water-based storage at 4 °C, 25 °C, and 37 °C to reflect relevant physiological and formulation conditions (**Figure 4.5**), enabling assessment of temperature-driven stability without ionic interference. Future work will investigate additional formulation and environmental stressors-including freeze–thaw, lyophilization, and agitation-, along with evaluations of drug-loaded silk nanoparticles at clinically relevant concentrations and with incorporated therapeutic agents to better assess their stability and suitability for drug delivery applications.

Chapter 5 Drug Loading and Drug Release of Doxorubicin-Loaded Silk Nanoparticles: A Comparison of Antisolvent Precipitation and Adsorption Methods



Chapter 5 demonstrates how the physicochemical properties of biomimetic silk nanoparticles are influenced by doxorubicin encapsulation via two different encapsulation methods. Using analytically distinct characterization techniques, the study provides a preliminary understanding of how manufacturing conditions shape pharmacologically relevant nanoparticle properties, especially drug encapsulation and release behaviour.

5.1. Introduction

Cancer remains a major global health challenge, with incidence and mortality continuing to rise. In Europe, cancer accounts for approximately 22% of new diagnoses and 20% of all deaths.³⁰⁷ Among existing chemotherapeutic agents, doxorubicin (DOX) is widely recognised for its broad-spectrum efficacy against breast cancer,^{308, 309} ovarian cancer,³¹⁰ colorectal cancer,³¹¹ cervical cancer,³¹² prostate cancer,^{313, 314} and liver cancer,³¹⁴ However, conventional DOX treatment is associated with cumulative dose-dependent cardiotoxicity and the emergence of chemoresistance mechanisms, prompting the development of alternative delivery strategies to improve therapeutic index and evade chemoresistance.³¹⁵

Nanomedicine has emerged as a promising approach to address these limitations, with PEGylated liposomal doxorubicin (PLD) representing a benchmark nanomedicine. First approved by the FDA in 1995 for Kaposi sarcoma, PLD demonstrated high efficacy at low doses (10–20 mg/m²) and enhanced tumour accumulation *via* the EPR effect. A subsequent phase III trial reported a 30% reduction in mortality, leading to its approval for ovarian cancer in 2001.¹⁵⁶ Another candidate is PEGylated liposomal doxorubicin (Doxil®, Janssen Pharmaceuticals), which offers reduced cardiotoxicity, prolongs drug and tumour exposure compared to free doxorubicin.^{2, 157, 158} However, significant concerns persist regarding the adverse effects of PLD-based, conventional, and non-PLD formulations, driving the ongoing need for alternative delivery strategies to improve patient outcomes.¹⁵⁹

Silk-based nanoparticles have gained attention as a versatile platform for drug delivery with their appeal stemming from their highly tunable physicochemical properties, compatibility with scalable processing, and their potential favourable pharmaceutical characteristics,³¹⁶⁻³¹⁸ including controlled drug release;^{101, 296} enhanced cellular internalization; improved solubility;^{101, 131} and increased therapeutic efficiency.^{137, 164, 172, 296, 319} Despite recent progress, the translation of silk biomaterials into efficient anticancer drug delivery systems

remains challenging, such as high drug loading and controlled release formulations, large-scale production, and long-term safety evaluations of silk nanoparticles are still under investigation.^{176-178, 184}

This chapter aims to explore the application of silk nanoparticles as carriers for the anticancer drug doxorubicin as a model drug. Doxorubicin-loaded silk nanoparticles were prepared using two distinct encapsulation methods: direct antisolvent precipitation and adsorption to compare differences in drug loading efficiency and particle-related parameters. A range of orthogonal analytical techniques were used to measure particle physicochemical properties and early translational potential for doxorubicin delivery focusing on , formulation stability, drug loading efficiency, and pH-triggered drug release. Moreover, the dose-dependent sensitivity of cells in response to exposure to placebo and doxorubicin-loaded silk nanoparticles was evaluated in RAW 264.7,MDA-MB-231, and DU 145 cells. These findings are expected to lay the foundations for the design of controllable silk-based drug nanocarriers and their downstream pre-clinical evaluation.

5.2. Materials and Methods

5.2.1. Materials

All reagents and solvents used for silk nanoparticle fabrication were purchased from Sigma-Aldrich or Acros Organics (>98% purity) and used without further purification unless otherwise noted. Doxorubicin hydrochloride was purchased from LC Laboratories, MA, USA. Reagents and chemicals not specifically mentioned in the text for cell culture experiments were obtained from Gibco™ (Thermo Fisher Scientific). *B. mori* silk cocoons were purchased from Scientific Center on Sericulture, Vratsa, Bulgaria.

5.2.2. Aqueous Silk Fibroin Preparation

The silk solution was prepared from *Bombyx mori* cocoons, as described elsewhere.^{23, 245} *B. mori* cocoons were boiled in Na₂CO₃ for 1 h, and the degummed silk was air-dried overnight. Dissolution was performed in LiBr at 60 °C for 4 h, followed by 48 h of dialysis against deionized water using a 3.5 kDa MWCO dialysis cassette (Slide-A-Lyzer 3.5K Dialysis Cassette G2, Thermo Scientific, Rockford, IL, USA). Centrifugation (2,885g, 40 min at 4 °C) (PK 121R Centrifuge, rotor T515, ACL International Srl, Milan, Italy) was conducted to collect the purified silk solution. The concentration of silk nanoparticles (% w/v) was determined from the dried weight aliquots incubated at 60 °C for 24 h.

5.2.3. Drug-Loaded Silk Nanoparticle Manufacture

Doxorubicin-loaded silk nanoparticles (DOX-SNPs) were manufactured using antisolvent precipitation and adsorption-based methods.

Antisolvent precipitation method

DOX-SNPs were manufactured using a direct antisolvent precipitation method, where the DOX solution was mixed with liquid SF. Antisolvent precipitation of SNPs was carried out by adding 3% (w/v) silk fibroin (6 mL) containing (0, 150, 300, and 600 µM) doxorubicin dropwise into IPA (30 mL). Final concentrations of silk fibroin was 0.5% w/v and doxorubicin in each batch were 0.5% and 0, 25, 50, and 100 µM to achieve different drug loading levels. SNP suspensions were centrifuged at 48,400g for 2 h at 4 °C, and the supernatant was transferred to a new container. The remaining drug in supernatant was assayed at 480 nm for doxorubicin using UV-Vis spectroscopy, pipetting 150 µL per well (Polarstar Omega, BMG Labtech, Ortenberg, Germany). Doxorubicin concentration was assayed according to a standard curve (0–100 µM, Abs: 480 nm). The remaining SNP pellet was washed twice more by adding 20 mL of distilled water, followed by sonication (30% amplitude, 30 s), topped up to 36 mL, and centrifugation. The production yield was calculated as described elsewhere.²⁴⁵

Adsorption method

Placebo SNPs were mixed with doxorubicin solution to yield SNP mixture (2 mL), containing 5 mg/mL SNPs and 0, 25, 50, and 100 μ M DOX. The mixtures were incubated at 4 °C with 30 oscil/min for 18 h (PMR-30 Platform 2D Rocker–Fixed Tilt, Grant Instruments (Cambridge) Ltd, UK), then centrifuged at 48,400 g at 4 °C for 2 h to collect the doxorubicin-loaded SNP (DOX-SNP). Doxorubicin concentration in the supernatant was quantified using UV-vis spectroscopy (Abs: 480 nm). The remaining SNP pellet was resuspended in distilled water, sonicated (30% amplitude, 30 s), and stored at 4 °C for further analyses. The drug encapsulation efficiency and drug loading content were calculated by the following equations.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Total added amount of DOX (mg)} - \text{Amount of DOX in supernatant (mg)}}{\text{Total added amount of DOX (mg)}} \times 100 \quad \text{Equation 5.1}$$

$$\text{Drug loading content (\mu g/mg)} = \frac{\text{Total added amount of DOX (\mu g)} - \text{Amount of DOX in the supernatant (\mu g)}}{\text{Nanosphere weight (mg)}} \quad \text{Equation 5.2}$$

5.2.4. Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS)

Particle size and zeta potential were measured using the DLS and ELS, respectively. The SNP suspension was diluted in deionized water at a 1:25 ratio for DLS measurement and a 1:20 ratio for ELS measurement. The measurement methods were set up as described in previous chapters.²⁴⁵

5.2.5. Field Emission Scanning Electron Microscopy (FE-SEM)

An aliquot of DOX-SNP (20 mL of 0.1 mg/mL) suspension was dropped onto a silicon wafer. Next, samples were air-dried at ambient temperature for 24 h, and gold coated using a gold sputter coater; 35 mm height, 40 s, 0.08 mb, and 30 mA (Agar Scientific Manual Sputter Coater, Agar Scientific Ltd., Essex, UK) and imaging via FE-SEM (Hitachi SU6600, Hitachi High-Tech Europe GmbH, Krefeld, Germany) with magnifications of 10,000 $\times$, 20,000 $\times$, and 60,000 $\times$ at 5 kV.

5.2.6. Lyophilization of Doxorubicin-Loaded Silk Nanoparticles

Freeze-dried doxorubicin-loaded silk nanoparticles were prepared by pre-freezing a 5 mg/mL SNP suspension at $-20\text{ }^{\circ}\text{C}$ overnight, followed by freeze-drying at $-10\text{ }^{\circ}\text{C}$ and 0.14 mbar for 24 hours. The freeze-dried SNPs were used for subsequent Fourier-Transform Infrared (FTIR) Spectroscopy analysis.

5.2.7. Fourier-Transform Infrared (FTIR) Spectroscopy

The secondary structures of silk and silk nanoparticles were analyzed using FTIR spectroscopy.²⁴⁵ The air-dried silk film and 70% ethanol-treated silk film served as the silk I and silk II references. FTIR spectra were recorded over $400\text{--}4000\text{ cm}^{-1}$ (4 cm^{-1} resolution) with an ATR-equipped TENSOR II spectrometer (Bruker Optik GmbH, Ettlingen, Germany), using 32 scans for the background and 128 for samples. Secondary structure content was determined from deconvoluted amide I spectra, assigning peaks to specific structures: antiparallel β -sheet ($1697\text{--}1710\text{ cm}^{-1}$), β -turn ($1663\text{--}1696\text{ cm}^{-1}$), random coil ($1638\text{--}1662\text{ cm}^{-1}$), native β -sheet ($1620\text{--}1635\text{ cm}^{-1}$), and intermolecular β -sheet ($1605\text{--}1625\text{ cm}^{-1}$).²³ Second-derivative amide I spectra ($1600\text{--}1700\text{ cm}^{-1}$) were used for determining correlation coefficient calculations against air-dried silk films.

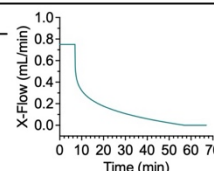
5.2.8. Frit-Inlet Asymmetric Flow Field-Flow Fractionation (FI-AF4)

Doxorubicin-loaded silk nanoparticles (DOX-SNPs) were separated using a Frit-inlet Asymmetric Flow FFF system (AF2000, PostNova Analytics, Landsberg am Lech, Germany). The regenerated cellulose membrane, MWCO of 10 kDa (Postnova AF2000 MF-AF4 Analytic RC Membrane, Z-AF4-MEM-618-10kDa, Postnova Analytics, Landsberg am Lech, Germany) was used as the accumulation wall for all measurements with a spacer thickness of $350\text{ }\mu\text{m}$. The in-line detectors multiplexed with FI-AF4 included a 21-multi-angle light scattering (MALS) detector (#PN3621, Postnova Analytics), a UV detector (#SPD-M40, Postnova

Analytics), a fluorescence detector (Ex/Em = 480/594 nm) (#FLD, Postnova Analytics), and an online Zetasizer Nano ZS (Malvern Panalytical). The DOX-SNPs (0.5 mg/mL) were dispersed in μ -filtered Milli-Q water as the carrier liquid. The separation method (67 min) was adapted with minor modifications from³²⁰ (**Figure 5.1a**). The direct injection method (20 min) was carried out for the determination of percent recovery and the doxorubicin calibration curve (0.1–100 μ M) (**Figure 5.1b**). The separation principle of FFF alongside FI-AF4 separation conditions is highlighted in **Figure 5.1c**.

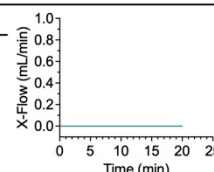
a Frit-inlet AF4 method

General setting	Elution step				Rinse step
Detection flow: 0.3 mL/min	Delay time: 2 min	Time (min)	X-Flow (mL/min)	Elution type	Tip pump: 0.3 mL/min
Spacer: 350 μ m	Injection flow: 0.2 mL/min	7	0.75	Constant	Time: 5 min
Run time: 67 min	Run time: 62 min	50	0.75	Power	
Solvent: 35.8 mL		5	0	Constant	
Injection volume: 20 μ L					



b Direct injection method (for recovery study & doxorubicin calibration curve)

General setting	Elution step				Rinse step
Detection flow: 0.5 mL/min	Delay time: 5 min	Time (min)	X-Flow (mL/min)	Elution type	Tip pump: 0.5 mL/min
Spacer: 350 μ m	Injection flow: 0.5 mL/min	15	0	Constant	Time: 5 min
Run time: 20 min	Run time: 15 min				
Solvent: 14.1 mL					
Injection volume: 20 μ L (SNP) 10 μ L (Doxorubicin)					



c Frit-inlet AF4 separation and detection

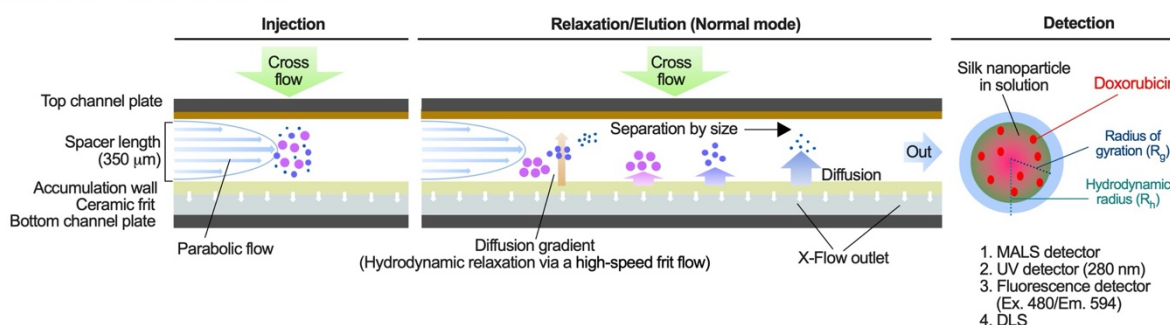


Figure 5.1 Schematic of silk nanoparticle characterisation using the frit-inlet asymmetric flow field-flow fractionation (FI-AF4). The 0.5 mg/mL doxorubicin-loaded silk nanoparticle was dispersed in 0.1 μ m-filtered Milli-Q water: **(a)** the FI-AF4 method used for the doxorubicin-loaded silk nanoparticle separation and characterisation, **(b)** the FI-AF4 direct injection method used for the recovery determination and the doxorubicin calibration curve, **(c)** the channel geometry, separation principle, and orthogonal detectors, including a multiangle light scattering (MALS) detector, a UV detector (DAD), a fluorescence detector (FLD), and a dynamic light scattering (DLS).

5.2.9. Single Particle Automated Raman Trapping Analysis (SPARTA) measurements and spectral analysis

Population heterogeneity of doxorubicin-loaded silk nanoparticles compared to empty silk nanoparticles was assessed using Single Particle Automated Raman Trapping Analysis (SPARTA) (SPARTA[®] AGIS I, SPARTA Biodiscovery Ltd, London, UK). The SPARTA Raman micro-spectroscopy system has been described elsewhere.³²¹ Briefly, the setup comprised a Raman micro-spectroscope (alpha300R⁺, WITec, Ulm, Germany); equipped with a 786 nm laser light source (Toptica XTRA II) and a 63 × /1.0 NA water immersion objective lens (W Plan-Apochromat, Zeiss, Oberkochen, Germany). Scattered light was collected using a UHTS 300 spectrometer (WITec, Ulm, Germany), and spectra were acquired at a spectral resolution of 3 cm⁻¹ with an 85 mW laser power (iDus DU401-DD, Andor, Belfast, UK).

Following synthesis *via* the adsorption method, silk nanoparticles were dispersed in 0.1 μM-filtered Milli-Q water to achieve a final concentration of 2.5 mg/mL of SNP. An aliquot of SNP (200 μL) was transferred to a 22 mm diameter coverslip with the blank (0.1 μM-filtered Milli-Q water), and analysed using a water-immersion lens. For each replicate, 300 individual trapped particles were trapped and analysed for 10 s, with a 1 s laser shutter off interval. The average of 50 blank spectra were used to perform background subtraction. Data processing and analysis were performed using the Discovery software (Release 1.2.1) (SPARTA[®] AGIS I, SPARTA Biodiscovery Ltd, London, UK).

5.2.10. *In Vitro* Drug Release

Cumulative release of doxorubicin was performed at 37 °C over 15 days. Briefly, 1.5 mg of doxorubicin-loaded SNPs formulated at a doxorubicin concentration of 100 μM (100 DOX-SNPs) were dialyzed against 1×PBS (pH 7.0), HEPES buffer (pH 6.5), and citrate phosphate buffer (pH 4.5) using a dialysis tube (Slide-A-Lyzer[™] MINI Dialysis Devices, 3.5K MWCO, Thermo Fisher Scientific, UK). Dialysis was carried out at 37 °C with continuous stirring at

400 rpm using a Variomag Multipoint (Thermo Fisher Scientific, UK). At pre-determined timepoints (1, 2, 3, 6, 24, 48, 72, 144, 216, and 360 h), 500 μ L aliquots were removed to the dissolution media and transferred to a microtube for absorbance readings (480 nm), while 500 μ L of pre-warmed fresh buffer was added to maintain a batch volume of 2 mL. All procedures were conducted under sterile conditions. Empty nanoparticles (0DOX-SNPs) were also processed in parallel as controls, and cumulative drug release was calculated by subtracting the release from 0DOX-SNPs. Release kinetics of 100DOX-SNPs were fit to four different kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer-Peppas models (Table 5.1).

Table 5.1 Kinetic models used for fitting the release kinetics of doxorubicin from silk nanoparticles. F indicates the % cumulative drug release up to time t. The k_0 , k_1 , k_H , and k are the kinetic constants derived from the mathematical models, which indicate the rate of drug release over time ($n=3$).^{322, 323}

Kinetic model	Equation	Plot	Model explanation
Zero-order	$F = k_0 t$	F vs t	Describe a slow released drug with a constant concentration and without disaggregation.
First-order	$\ln(1-F) = -k_1 t$	$\ln(1-F)$ vs t	Describe a drug dissolution that its concentration is a direct correlation to its release rate.
Higuchi	$F = k_H \sqrt{t}$	F vs $\sqrt{t}$	Describe a controlled drug release from the matrix according to a water-solubility, resulting from a swelling and/or a degradation of the matrix.
Korsmeyer-Peppas	$F = k t^n$	$\log(F)$ vs $\log(t)$	Describe a drug release from the polymeric matrix (e.g., spherical nanoparticles, films, tablets), resulting from a water diffusion into matrix, matrix swelling, and matrix dissolution. This model is fitted to the first 60% drug release data.

5.2.11. *In vitro* Cytotoxicity of Doxorubicin-Loaded Silk Nanoparticles

The dose-dependent cytotoxicity of doxorubicin-loaded silk nanoparticles (DOX-SNPs) was examined in DU 145 (prostate) and MDA-MB-231 (triple-negative breast cancer) cancer cell lines, and RAW 264.7 murine macrophage (ATCC, product number TIB-71TM, ATCC, UK). DU 145 and MDA-MB-231 cells were cultured in complete RPMI 1640 media (10% v/v FBS, 50 U/mL penicillin, 50 mg/mL streptomycin, 2 mM sodium pyruvate, and 1% v/v RPMI amino acids). RAW 264.7 cells were cultured in complete DMEM media (10% v/v FBS, 50 U/mL llin, 50 mg/mL streptomycin, 2mM L-alanyl-L-glutamine dipeptide (Gibco™ GlutaMax™,

Thermo Fisher Scientific, UK). Cells were plated into a 96-well plate to obtain a cell density of 5000 cells/cm² for DU 145, 9375 cells/cm² for MDA-MB-231, and 15000 cells/cm² for RAW 264.7, and incubated overnight in a 5% CO₂ incubator at 37 °C before treatment with silk nanoparticles. Test concentrations used ranged between 0.1–1500 µg/mL for SNPs and 0.001–1000 µg/mL for doxorubicin-loaded silk nanoparticles prepared by the adsorption method (DOX-SNP-Ad). A concentration range of 0.001–100 mM doxorubicin was used to determine the dose-dependent cytotoxicity of the payload in the absence of SNPs. Next, cells were incubated for 48 h with growth media containing test samples, and cell viability was measured using the CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega UK Ltd., UK). The luminescence signal was measured using an integration time of 0.3 sec/well in a Spark[®] Multimode Microplate Reader (Tecan Trading AG, CH).

5.2.12. Cell Uptake Assay and Intracellular Doxorubicin Measurement

In this study, MDA-MB-231 cells were selected as a model. Cells were plated into a 12-well plate at cell density mentioned above. Cells were cultured in complete media for 24 h, before being exposed to fresh serum-free media containing doxorubicin-loaded silk nanoparticles prepared by the adsorption method (DOX-SNP-Ad) at a concentration of 0.5×EC₅₀ and 1×EC₅₀ value. The serum-free media served as a negative control. The 0.5×EC₅₀ doxorubicin (DOX) served as a free drug control. For fixed cell imaging, the cells were allowed to expose with doxorubicin-loaded silk nanoparticles for 0.5, 1, and 4 h. Cells were fixed with 4% v/v paraformaldehyde, stained with 1 µg/mL DAPI (Ref 62248, Thermo Scientific, UK), and imaged using a confocal microscope (Leica SP8, Leica Microsystems Ltd, UK). The mean intensity ratio of DOX/DAPI and nuclei circularity index were computed by a particle analysis function of Fiji/ImageJ.^{324, 325}

For intracellular doxorubicin quantification, cells were exposed to doxorubicin-loaded silk nanoparticles for 0.5–24 h. Cells were washed with cold 1×PBS before adding 500 µL of 1% v/v Triton X-100. After 30 min of cell lysis, the plates were shaken for 5 min at 600 rpm (Stuart Microplate Shaker SSM5, The Lab World Group, USA). Intracellular doxorubicin was quantified by measuring fluorescence intensity (Ex/Em = 480/594 nm) (Spark[®] Multimode Microplate Reader, Tecan Trading AG, CH).

5.3. Results

5.3.1. Silk Nanoparticle Physicochemical Property

All silk nanoparticles were manufactured using the antisolvent precipitation method. Two groups of doxorubicin-loaded silk nanoparticles (DOX-SNPs) were prepared. In the first group, doxorubicin was encapsulated during silk assembly *via* antisolvent precipitation and was designated as “DOX-SNP-ASP,” indicating preparation by the antisolvent precipitation method. In the second group, doxorubicin was adsorbed onto the surface of preformed silk nanoparticles and was designated as “DOX-SNP-Ad,” indicating preparation by the adsorption method. These two formulations were prepared to investigate and compare their pharmaceutical performance in terms of drug encapsulation efficiency and release behaviours.

The physicochemical properties of placebo and DOX-SNPs were evaluated using DLS, ELS, and FE-SEM, along with measurements of drug loading and encapsulation efficiency. The adsorption approach to doxorubicin encapsulation resulted in a significant increase in SNP particle size (91 d.nm) at the highest doxorubicin concentration (100 µM). In contrast, the antisolvent precipitation method yielded SNPs with increasing particle size as a function of doxorubicin content, ranging from 85–92 nm. Particle size measurements indicate no significant difference between the two methods for doxorubicin encapsulation (50–100 µM)

(**Figure 5.2a**). For the PDI values, larger variations were observed with the adsorption method; however, no significant differences were detected between different encapsulation methods and across different doxorubicin concentrations (**Figure 5.2b**). A pronounced drop in ZP was noted with the antisolvent precipitation method, decreasing from -34 to -28 mV, whereas the adsorption method had minimal impact on ZP, with values ~ -30 mV (**Figure 5.2c**).

The impact of manufacturing methods on drug encapsulation was assessed by measuring residual doxorubicin in the supernatant after collecting doxorubicin-loaded silk nanoparticles. The adsorption method exhibited a greater influence on drug loading, with encapsulation efficiency $<90.47\%$ at the highest doxorubicin concentration ($100\text{ }\mu\text{M}$ versus 64.57% for the nanoprecipitation approach) (**Figure 5.2d**). In contrast, drug loading content determined from the antisolvent precipitation method was 3.4-fold higher than the adsorption method (**Figure 5.2e**), with an increased yield corresponding to higher doxorubicin concentrations (**Figure 5.2f**).

Circularity determined from FE-SEM micrographs demonstrated circularity values close to 1, indicating a circular shape as well as shape retention after doxorubicin encapsulation *via* both antisolvent precipitation and adsorption methods. SEM micrograph showed a uniformity in size with less variation occurred as doxorubicin concentration increase: ~ 70 d.nm for the antisolvent precipitation method (**Figure 5.3a**) and 77 to 70 d.nm for the adsorption method (**Figure 5.3b**). Additionally, the size computed from the SEM micrograph tended to be smaller than the value reported by DLS.

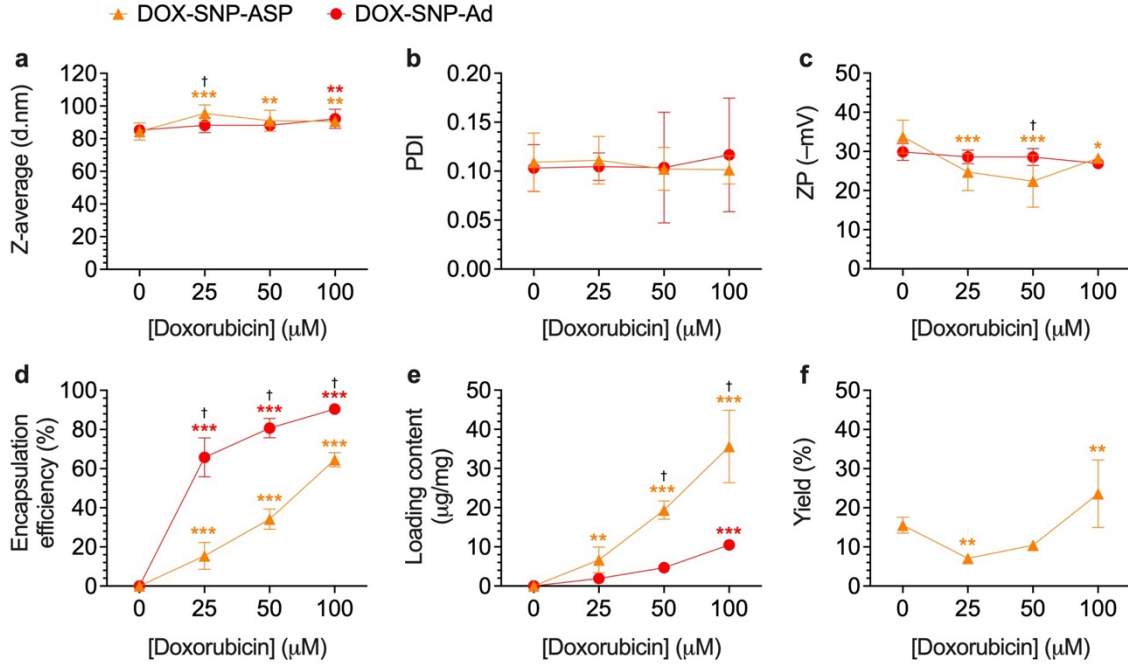


Figure 5.2 Physicochemical properties of doxorubicin-loaded silk nanoparticles (DOX-SNPs) manufactured by the antisolvent precipitation (DOX-SNP-ASP) and adsorption (DOX-SNP-Ad) methods, using 0–100 μM doxorubicin concentrations. (a, d) Particle size (Z-average) and (b, e) PDI, (c, f) ZP. Two-way ANOVA and Tukey's multiple comparisons test were used for statistical analyses: * indicates the significant difference of each variable from the 0DOX-SNP within the same group: $p < 0.1$ (*), $p < 0.01$ (), $p < 0.001$ (***) (ASP: pink; Ad: red); $\dagger$ indicates the significant difference of each variable of the same SNP species from the antisolvent precipitation method: $p < 0.1$ ($\dagger$), $p < 0.01$ ($\dagger\dagger$), $p < 0.01$ ($\dagger\dagger\dagger$). All experiments were conducted as three independent formulation batches with three technical repeats each ($n = 3$).**

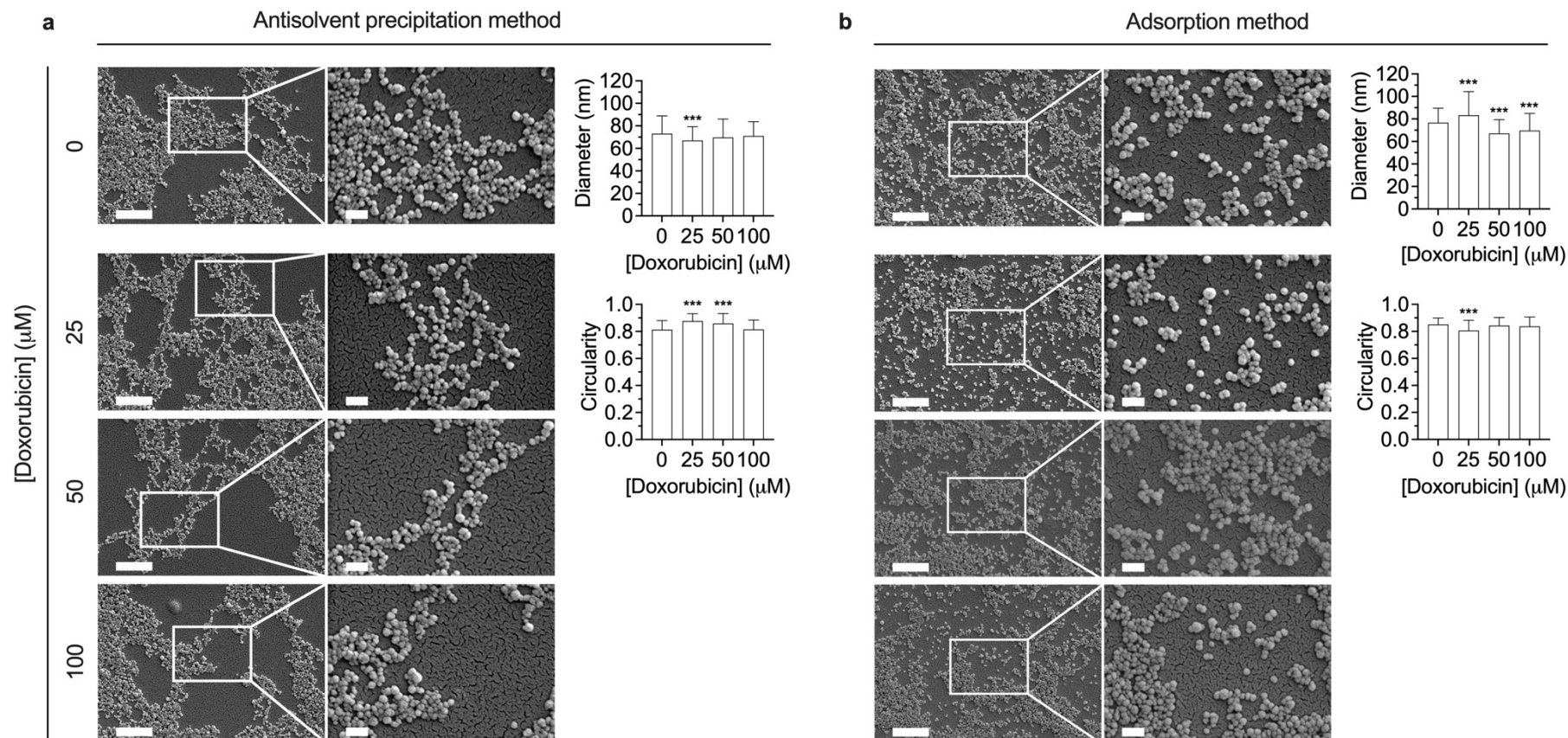


Figure 5.3 FE-SEM micrographs and computed particle size and shape of doxorubicin-loaded silk nanoparticles (DOX-SNPs), manufactured by (a) the antisolvent precipitation (DOX-SNP-ASP) and (b) the adsorption (DOX-SNP-Ad) method with 0–100 μM doxorubicin. DOX-SNPs were prepared at 0.1 mg/mL in deionized water, air-dried at ambient temperature, and gold-coated. Computed diameter and circularity *via* Image J software, using ~400–500 particles. One-way ANOVA and Dunnett's multiple comparisons test were used for statistical analyses, and compared to 0 μM doxorubicin of each: $p < 0.1$ (*), $p < 0.01$ (**), $p < 0.001$ (***). FE-SEM imaging was conducted as one experimental repeat with three technical repeats for circularity and size calculation ($n = 3$). Ca^{2+} -mediated silk nanoparticle (Ca^{2+} -SNP); K^{+} -mediated silk nanoparticle (K^{+} -SNP).

5.3.2. Determination of Drug and Protein Secondary Structure Using Fourier-Transform Infrared (FTIR) Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was used for the secondary structure analysis of silk nanoparticles (**Figure 5.4**). Air-dried silk film and 70% ethanol-treated silk film served as the silk I and silk II references, representing low and high β -sheet content, respectively.

Silk I and silk II references exhibited a significant difference in overall secondary structure, with a β -sheet content of 41% (± 2) for silk I and 65% (± 4) for silk II. In contrast, silk I exhibited a higher proportion of random coil structures ($36\% \pm 2$) compared to the silk II reference ($13\% \pm 1$), while a similar degree of β -turn content was observed in both ($\sim 23\%$) (**Figure 5.5**). FTIR data indicate that the doxorubicin-loaded silk nanoparticles—fabricated by antisolvent precipitation and adsorption methods predominantly consisted of a β -sheet-rich structure ($\sim 60\%$), followed by a β -turn ($\sim 30\%$) and random coil ($\sim 10\%$) structures.

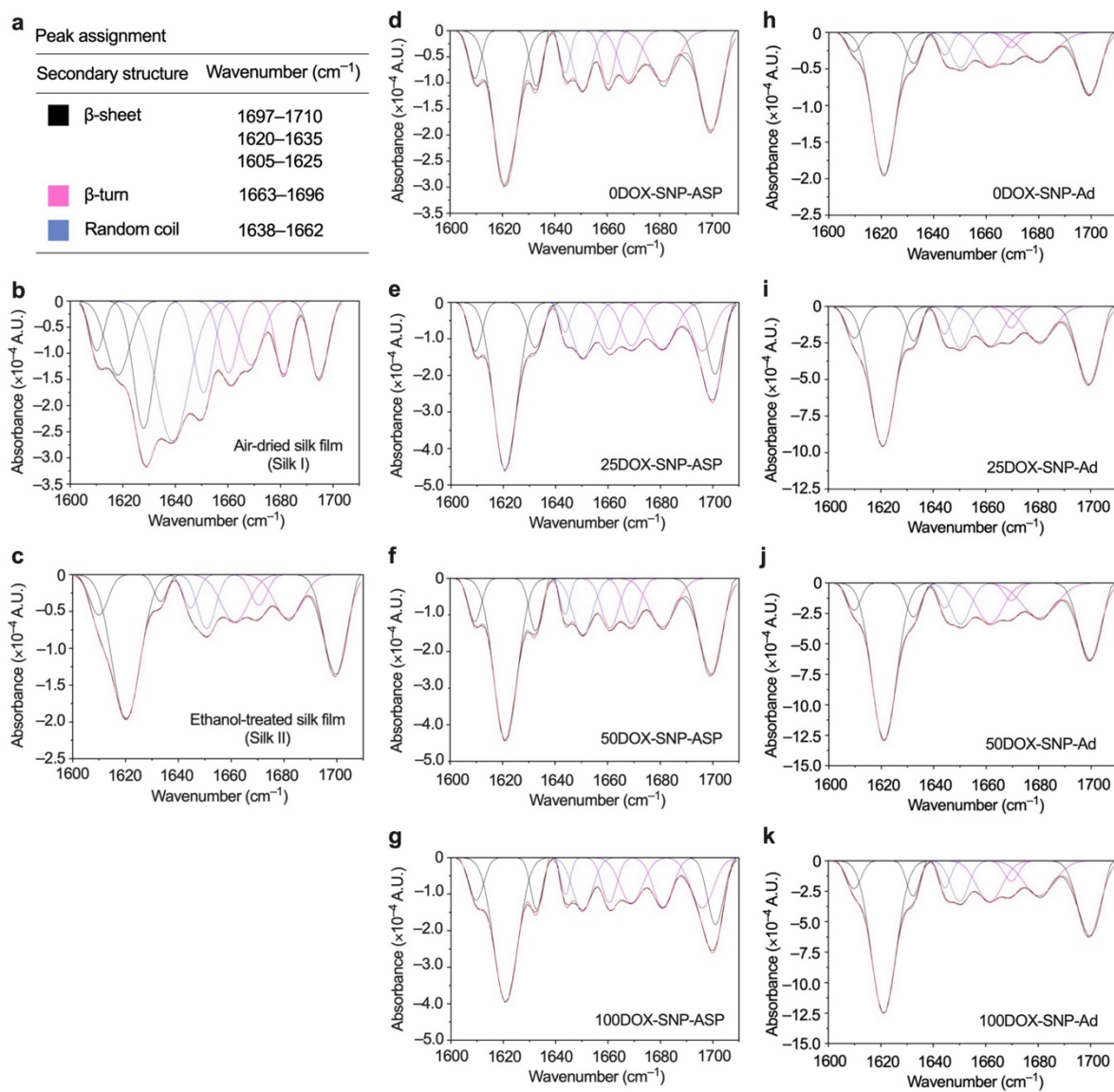


Figure 5.4 Exemplar IR fitting in the Amide I region (1700–1600 cm⁻¹). (a) IR peak assignment, (b) silk I and (c) silk II references, respectively. Doxorubicin-loaded silk nanoparticles manufactured by (d-g) antisolvent precipitation and (h-k) adsorption methods, containing 0–100 μ M doxorubicin. The experiment was conducted as three independent experimental repeats ($n = 3$).

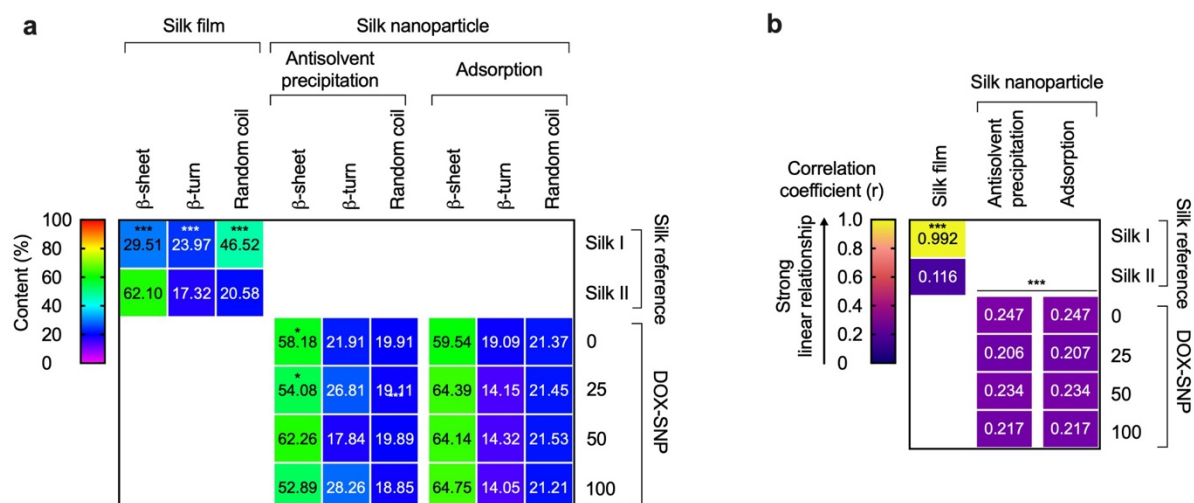


Figure 5.5 Silk nanoparticle secondary structure content. Secondary structure content was obtained from the deconvolution and calculation of IR spectra in the Amide I region ($1700\text{--}1600\text{ cm}^{-1}$): **(a)** structure content of silk film (Silk I and Silk II references) and doxorubicin-loaded silk nanoparticles (DOX-SNPs), manufactured by antisolvent precipitation and adsorption methods ($n = 3$); **(b)** correlation coefficient, using silk I as a reference. Two-way ANOVA and Dunnett's multiple comparisons test were used for statistical analyses: $p < 0.1$ (*), $p < 0.01$ (**), $p < 0.001$ (***). The experiment was conducted as three independent experimental repeats ($n = 3$).

5.3.3. Analysis of Doxorubicin-Loaded Silk Nanoparticles by Frit-Inlet Asymmetric Flow Field-Flow Fractionation (FI-AF4-MALS-UV-Fluorescence-DLS)

The fractograms of doxorubicin-loaded silk nanoparticles prepared by both antisolvent precipitation and adsorption methods were analysed using frit-inlet asymmetric flow field-flow fractionation (FI-AF4). The overlaid MALS, UV 280, and fluorescence signals are shown in **Figure 5.6**, detecting the silk nanoparticle signal *via* by MALS (**Figure 5.6a** and **Figure 5.6e**) and UV 280 detectors (**Figure 5.6b** and **Figure 5.6f**), while doxorubicin was detected *via* the fluorescence detector (**Figure 5.6c** and **Figure 5.6g**).

The elution profile for the antisolvent precipitation method displayed a single sharp peak (**Figure 5.6a–c**). In contrast, the adsorption method markedly altered the fractogram profile, significantly reducing the retention time and introducing two elution peaks that eluted before the cross-flow power decay (**Figure 5.6e–g** and **Table 5.2**). Doxorubicin-loaded nanoparticles showed considerably higher fluorescence signal intensity than placebo silk nanoparticles for both manufacturing methods (**Figure 5.6c** and **Figure 5.6g**).

Both the manufacturing method and doxorubicin loading significantly affected the MALS-measured R_g , while DLS-measured R_h was largely unaffected (**Table 5.2**). The antisolvent precipitation method produced the greatest deviation in shape factor from the ideal spherical value³⁰¹ (**Figure 5.6d**, **Figure 5.6h**, and **Table 5.2**). Additionally, both factors strongly influenced % recovery, which was higher with doxorubicin incorporation and best maintained using the adsorption method (**Table 5.2**).

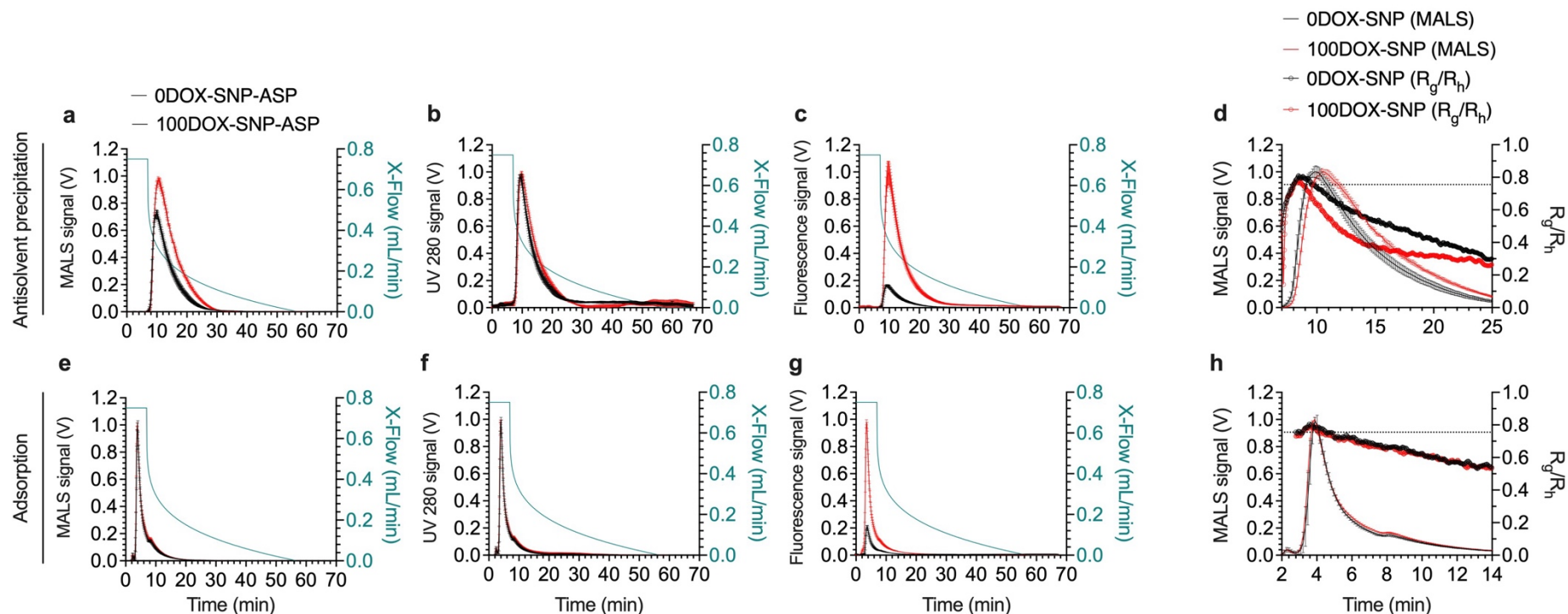


Figure 5.6 Frit-inlet asymmetric flow field-flow fractionation (FI-AF4) fractogram. FI-AF4 was conducted with a 20 μL injection volume of 0.5 mg/mL silk nanoparticle suspension, manufactured by antisolvent precipitation (upper panel) and adsorption (lower panel) methods. Filtered ultrapure water was used as the carrier liquid. The inline detectors, including (a, e) MALS (7° – 164°), (b, f) UV (280 nm), and (c, g) fluorescence detectors (Ex/Em = 480/594 nm), and online DLS, were used. The elution method was carried out by power decay of the Cross-Flow (X-Flow). (d, h) analysis of shape factor. FI-AF4-MALS trace aligned with computed shape factor (R_g/R_h). R_g and R_h were obtained from MALS and DLS detector measurements. The dotted line indicates an ideal spherical shape factor of 0.755.³⁰¹ The experiment was conducted as three technical repeats ($n = 3$).

Table 5.2 Retention time, particle radius, morphology, and recovery analysed by frit-inlet asymmetric flow field-flow fractionation (FI-AF4). Two-way ANOVA and Uncorrected Fisher's LSD were used for statistical analyses. The experiment was conducted as three technical repeats ($n = 3$).

Silk nanoparticle	Retention time (min)			Particle radius (nm)		Shape factor (R _g /R _h)	% Recovery
	MALS	UV 280	Fluorescence	MALS-R _g	DLS-R _h		
Antisolvent precipitation method							
0DOX-SNP-ASP	9.87 (±0.20)	9.42 (±0.34)	9.24 (±0.35)	28.82 (±0.07)	36.78 (±0.44)	0.784 (±0.008)	46.81 (±2.33)
100DOX-SNP-ASP	10.63 (±0.20)***	9.78 (±0.13)*	9.64 (±0.13)*	27.90 (±0.26)***	39.63 (±1.40)**	0.705 (±0.030)***	104.69 (±0.29)***
Adsorption method							
0DOX-SNP-Ad	3.88 (±0.13)†††	3.97 (±0.08)†††	3.66 (±0.08)†††	30.38 (±0.09)†††	38.15 (±0.48)	0.796 (±0.008)	83.31 (±1.18)†††
100DOX-SNP-Ad	3.88 (±0.00)†††	3.93 (±0.08)†††	3.53 (±0.08)†††	30.11 (±0.22)†††	38.74 (±0.42)	0.777 (±0.014)†††	89.94 (±0.74)***, †††

* indicates the significant difference of each variable from the 0DOX-SNP within the same manufacturing group: $p < 0.1$ (^{*}), $p < 0.01$ (^{**}), $p < 0.001$ (^{***}).

† indicates the significant difference of each variable between manufacturing methods: $p < 0.1$ ([†]), $p < 0.01$ (^{††}), $p < 0.001$ (^{†††}).

5.3.4. Single Particle Automated Raman Trapping Analysis (SPARTA) of Doxorubicin-Loaded Silk Nanoparticles (Adsorption Formulation)

The use of SPARTA was explored to evaluate the chemical composition of doxorubicin-loaded silk nanoparticles. Due to the enhanced drug release observed with the adsorption method, doxorubicin-loaded silk nanoparticles prepared via the adsorption method were selected for analysing doxorubicin entrapment. Doxorubicin-loaded silk nanoparticles (100DOX-SNP-Ad) exhibited significant differences in Raman signal intensity (421–1800 cm^{-1}), with increased intensity at characteristic doxorubicin Raman shifts of 440, 463, 1209, 1240, 1250, 1292, 1411, 1423, and 1576 cm^{-1} , compared to empty silk nanoparticles (0DOX-SNP-Ad) (**Table 5.3**, **Table 5.4**, and **Figure 5.7a**). Univariate analysis of the integrated Raman signals indicated a significant increase in doxorubicin content, with up to 95% of the particle population showing detectable loading at 1209 cm^{-1} (**Figure 5.7b–h**). In addition, the correlations among all integrated signals were illustrated, indicating high consistency in doxorubicin detection and demonstrating strong agreement in Raman signal intensity across the different Raman shifts (**Figure 5.8a–u**).

Table 5.3 Raman shift assignment for doxorubicin.

Doxorubicin characteristics ³²⁶	Raman shift (cm ⁻¹) (I ₀ = 785 nm)			Statistic method
	Reference 1 ³²⁶	Reference 2 ³²⁷	Experimental	Range (cm ⁻¹)
C–C–O	440*		440	437–446
C–O	462*	450		
C–C of ring	989*	465	463	460–467
C–O	1082*			
C–O–H	1209*	1205	1209	1200–1215
		1232		
C–H	1241*		1240	
		1247	1250	1233–1254
		1291	1292	1288–1296
Vibration in-plane of C–O and C–O–H; C–H bending	1299			
		1426	1411	
			1423	1408–1430
C=O and C–C stretching of the aromatic hydrocarbons	1448			
		1460		
Ring stretching	1578*	1577	1576	1570–1580
C=O	1637*	1644		

* indicates the crucial characteristic peaks of doxorubicin, as evidenced elsewhere.³²⁶

Table 5.4 Raman shift assignment for proteins.

Protein Characteristic (SPARTA and ³²⁸)	Raman shift (cm ⁻¹)
Protein primary peaks	1000, 1210–1275, 1425–1480, 1635–1695
Amide stretches	1210–1275, 1635–1695
Amide III (C–N) stretches	1200–1300
Amide I (C=O) stretches	1660
Phenylalanine/Tyrosine	1609
C–N stretches	1080
Phenylalanine	1000, 1003
C–C backbone stretches α -helix	937
Tyrosine	850
Tyrosine	825

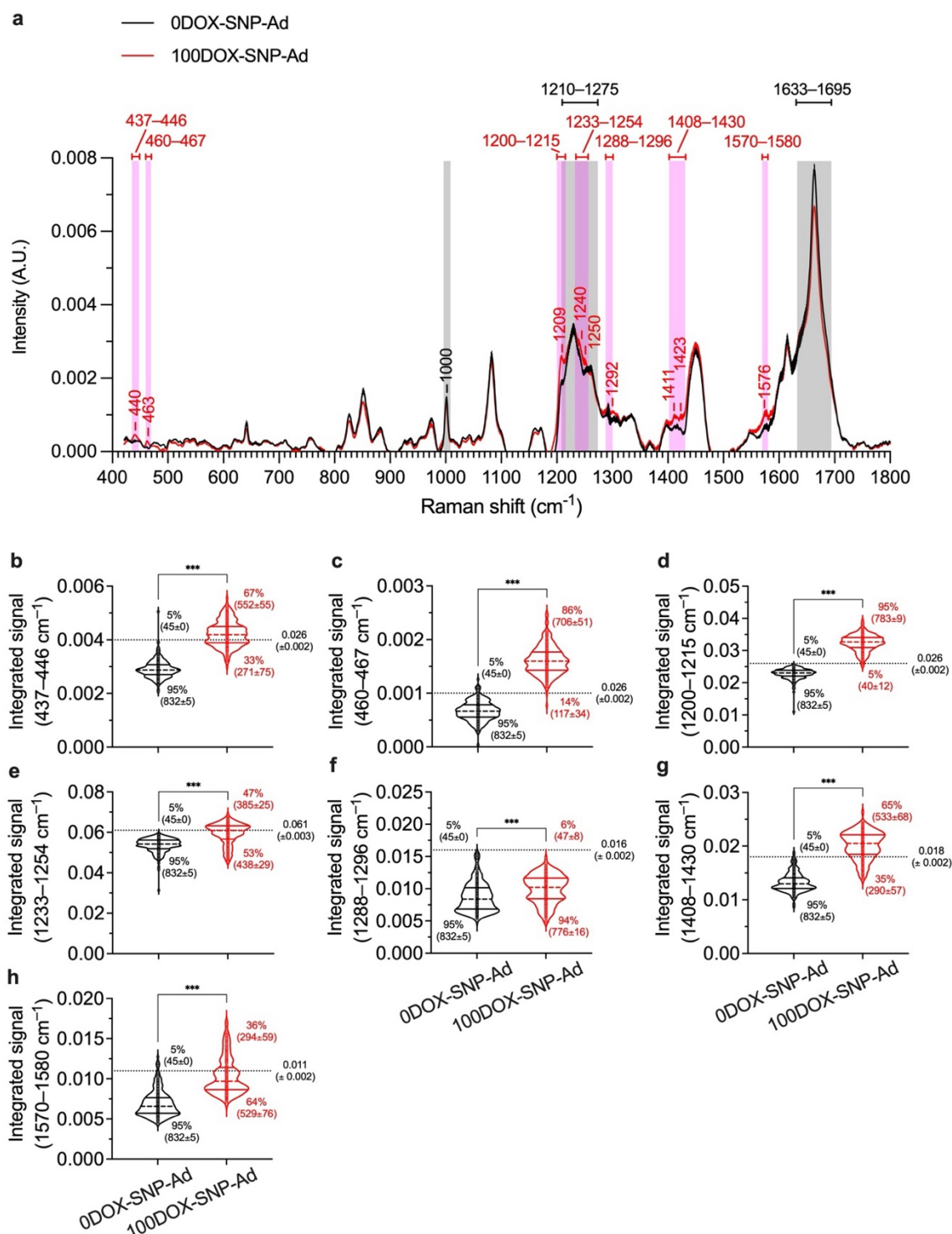


Figure 5.7 Single Particle Automated Trapping Analysis (SPARTA) of doxorubicin-loaded silk nanoparticles prepared by the adsorption method. An aliquot of 100 μ L silk nanoparticle suspension (0.25 mg/mL) was prepared in 0.1 μ m-filtered Milli-Q water, which also served as a blank. The measurement was performed in triplicate: 877 and 823 nanoparticles for 0DOX-SNP-Ad and 100DOX-SNP-Ad, respectively. **(a)** The overlay of Raman spectra (421–1800 cm^{-1}): the black colour indicates protein peaks; the pink colour indicates the doxorubicin peaks. **(b–h)** The integrated Raman signal analysed by the univariate analysis mode was determined in a range covering the identified peaks for doxorubicin (pink shading). An unpaired t-test was used for statistical analysis to compare between two groups (0DOX-SNP-Ad and 100DOX-SNP-Ad): $p < 0.1$ (*), $p < 0.01$ (**), $p < 0.001$ (***) . The experiment was conducted as three technical repeats ($n = 3$).

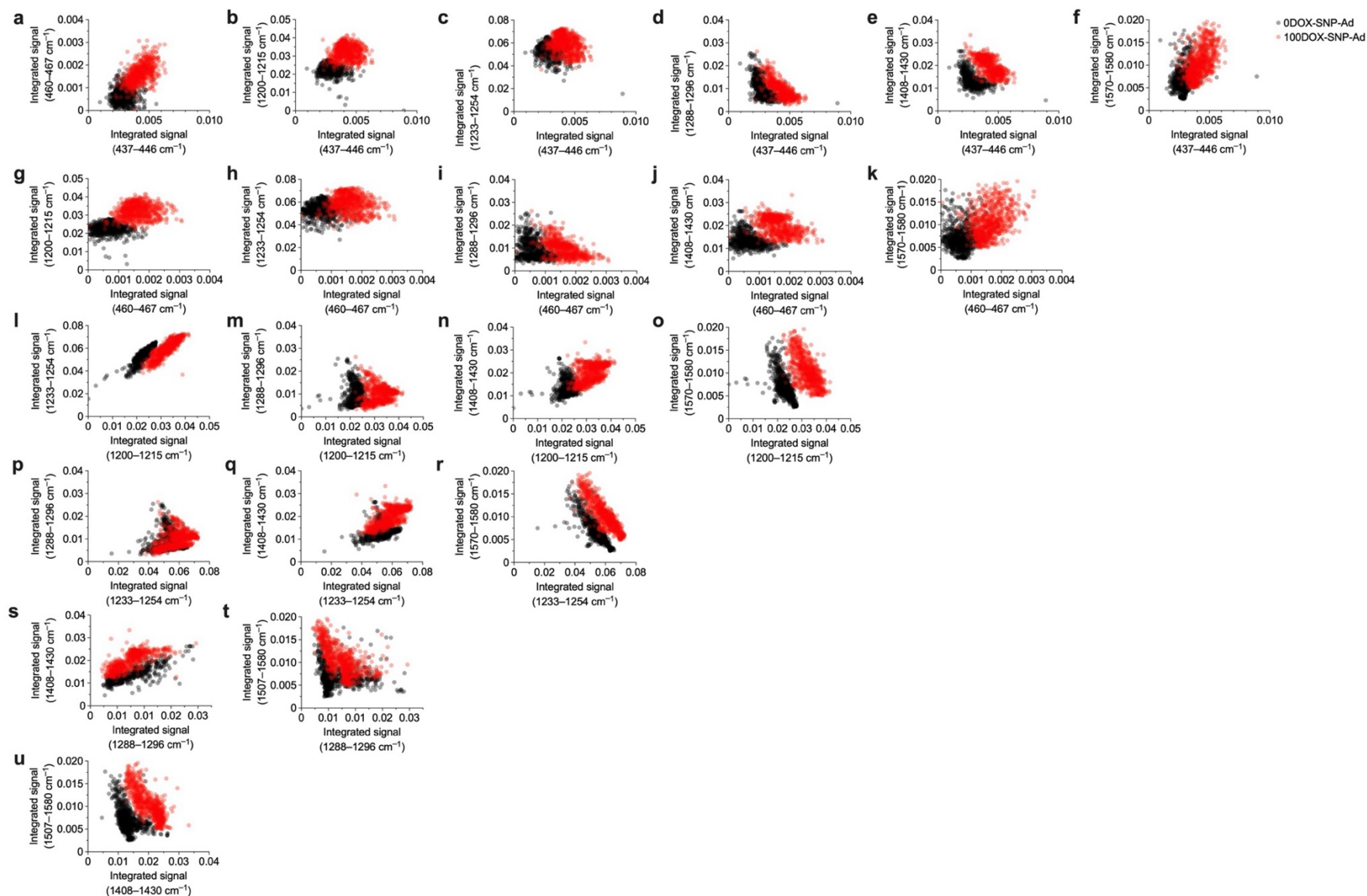


Figure 5.8 Bivariate analysis of doxorubicin-loaded silk nanoparticles prepared by the adsorption method using Single Particle Automated Trapping Analysis (SPARTA). An aliquot of 100 μL silk nanoparticle suspension (0.25 mg/mL) was prepared in 0.1 μm -filtered Milli-Q water and was used for analysis. The 0.1 μm -filtered Milli-Q water served as a blank. The measurement was performed in triplicate: 877 and 823 nanoparticles for 0DOX-SNP-Ad and 100DOX-SNP-Ad, respectively. Seven Raman shift ranges were integrated before bivariate analysis, showing a correlation with (a-f) 437–446 cm^{-1} , (g-k) 460–467 cm^{-1} , (l-o) 1200–1215 cm^{-1} , (p-r) 1233–1254 cm^{-1} , (s, t) 1288–1296 cm^{-1} , and (u) 1408–1430 cm^{-1} on the X-axis. The experiment was conducted as three technical repeats ($n = 3$).

5.3.5. Drug Loading Content and Heterogeneity of Drug-Loaded Particles

The incorporation of the fluorescence detector in the FI-AF4 method enabled the determination of drug loading based on the intrinsic fluorescence of doxorubicin ($\text{Ex/Em} = 480/594$), providing a comparison with drug adsorption measurements obtained by UV-Vis spectroscopy (**Figure 5.2e**). The AF4 direct-injection method revealed an initial drug content in the silk nanoparticles that differed from the UV-Vis results: 0.71-fold lower for the antisolvent precipitation method and 3.51-fold higher for the adsorption method. Interestingly, after separation, drug loss was observed for both methods; however, the antisolvent precipitation method (56.33%) retained a significantly higher amount of drug than the adsorption method (34.08%) (**Figure 5.5**).

The heterogeneous drug loading of doxorubicin-loaded silk nanoparticles, represented here by the adsorption method as a preliminary case study, was evaluated using the SPARTA technique. The analysis showed that drug-encapsulated particles accounted for approximately 95% of the total population (**Table 5.5**).

Table 5.5 Comparison of drug loading content studied with UV-Vis (480 nm) and FI-AF4-fluorescence (Ex/Em = 480/594 nm), and %Encapsulated particles studied by Single Particle Automated Raman Trapping Analysis (SPARTA) method. The antisolvent precipitation and adsorption methods influenced the encapsulation efficiency, drug loading content, and yield of doxorubicin-loaded silk nanoparticles. The unpaired t-test with Welch's correction was used for statistical analyses. The UV-Vis spectroscopy method was conducted as three independent formulation batches with three technical repeats each ($n = 3$), while Frit-inlet AF4-MALS-UV-FLD-DLS method and SPARTA method were conducted as three technical repeats ($n = 3$). Not available (NA)

Silk nanoparticle	UV-Vis spectroscopy method		Frit-inlet AF4-MALS-UV-FLD-DLS method			SPARTA method
	Encapsulation efficiency (%)	Drug loading content ($\mu\text{g}/\text{mg}$ DOX-SNP)	Drug loading content ($\mu\text{g}/\text{mg}$ DOX-SNP)		% Drug remaining	%Encapsulated particles examined at the major drug peak of 1209 cm^{-1}
			Direct injection	X-Flow method		
100DOX-SNP-ASP	64.57 (± 3.61)	35.61 (± 9.27)	25.59 (± 0.51)	14.41 (± 1.36)	56.33 (± 5.30)	NA
100DOX-SNP-Ad	90.47 (± 1.93) ^{†††}	10.49 (± 0.22) ^{†††}	36.86 (± 0.07) ^{†††}	12.56 (± 0.30)	34.08 (± 0.80) [†]	95 (± 4)

[†] indicates the significant difference of each variable between manufacturing methods: $p < 0.1$ ([†]), $p < 0.01$ (^{††}), $p < 0.001$ (^{†††}).

5.3.6. *In vitro* Drug Release

The dissolution was carried out at different pH levels, as predicting their release behaviour near the physiological pH of 7.4 (pH 7.0), tumour microenvironment (pH 6.5), and lysosomal environment (pH 4.5) (**Figure 5.9a-b**). An *in vitro* release kinetic study was performed for both SNP prototypes using four different kinetic fit models, including the zero-order, first-order, Higuchi, and Korsmeyer-Peppas models (**Figure 5.10** and **Figure 5.11**). Amongst these four models, the Higuchi and Korsmeyer-Peppas models showed the best goodness of fit, providing a $> 0.8 R^2$ value, for both prototypes (**Figure 5.9c**). At pH 4.5, the rate constant (k) according to the Higuchi model for doxorubicin-loaded silk nanoparticles changed from 0.34 to 1.31 $\text{h}^{-1/2}$ for the antisolvent precipitation method, and from 0.43 to 0.31 $\text{h}^{-1/2}$ for SNPs generated by the adsorption method. A corresponding increase in k was also observed for the Korsmeyer Peppas fit from 0.15 to 0.52 h^{-n} for the antisolvent precipitation method, and 0.543 to 1.15 h^{-n} for SNPs generated by the adsorption method (**Figure 5.9d**). The mode of drug release mechanism (n) calculated by the Korsmeyer-Peppas model indicated that SNPs generated from both methods exhibited an anomalous (non-Fickian) diffusion (**Figure 5.9e-f**).

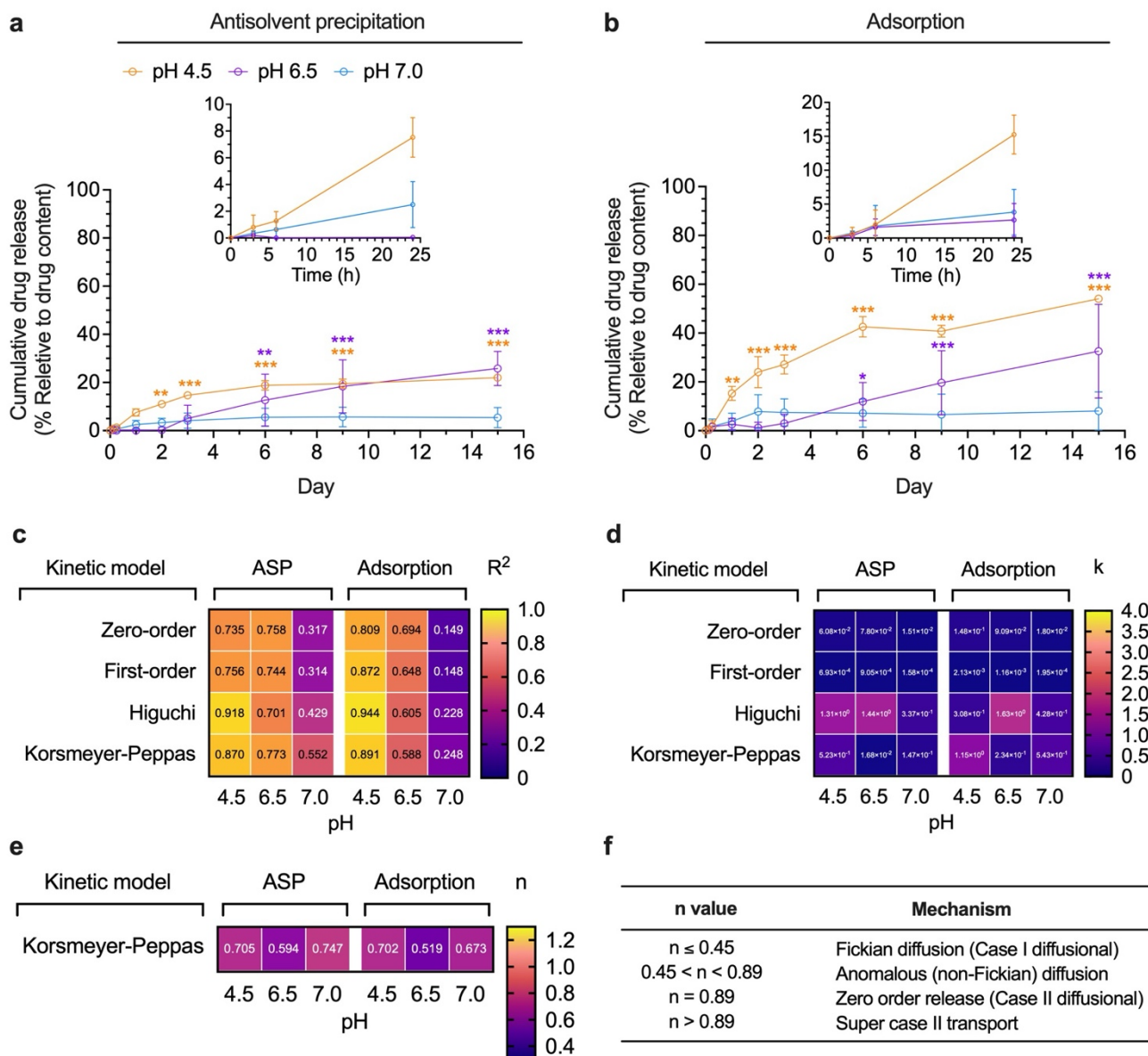


Figure 5.9. Cumulative *in vitro* drug release and kinetics. Doxorubicin-loaded silk nanoparticles (100DOX-SNPs) and empty silk nanoparticles (0DOX-SNPs) were prepared at 1.5 mg protein, followed by a dialysis against 2 mL of buffers (1×PBS pH 7.0, HEPES buffer pH 6.5, and citrate phosphate buffer pH 4.5) for 15 days. The dialysate (500 μ L) was aliquoted at time points for drug content determination, followed by a replacement of an equal volume of buffer. The 0DOX-SNP served as a baseline for subtraction. Two-way ANOVA and Dunnett's multiple comparisons test were used for statistical analyses: $p < 0.1$ (*), $p < 0.01$ (**), $p < 0.001$ (***). The experiment was conducted as three technical repeats ($n = 3$). The release kinetics: **(c)** The R-squared (R^2) denotes the goodness of fit of the model: a higher R^2 indicates a better fit;³²⁹ **(d)** The rate constant (k) denotes the rate of drug release over time;³³⁰ **(e)** The exponent (n) denotes the mode of drug release mechanism calculated by the Korsmeyer-Peppas model; **(f)** the interpretation of the n value.³²²

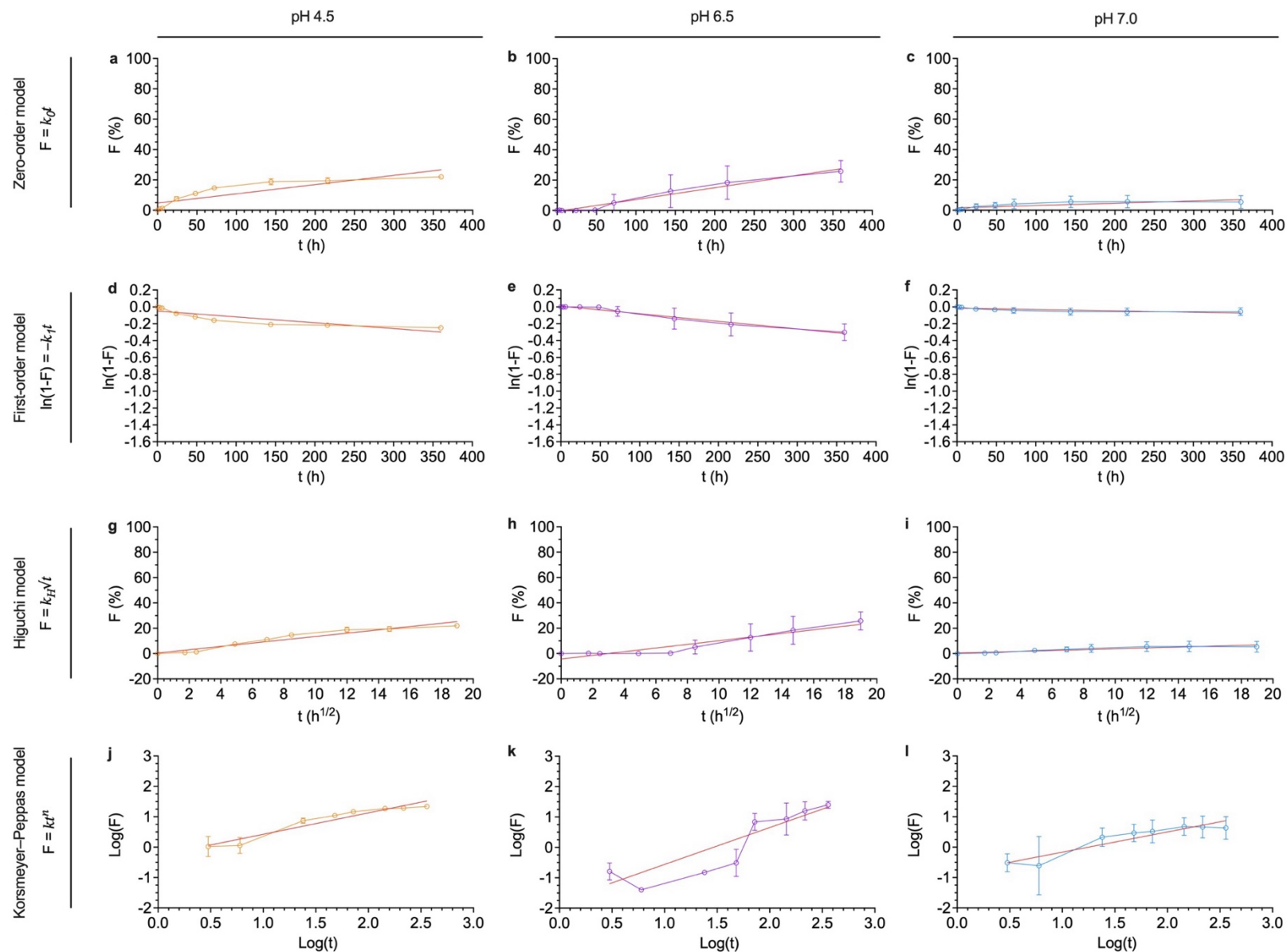
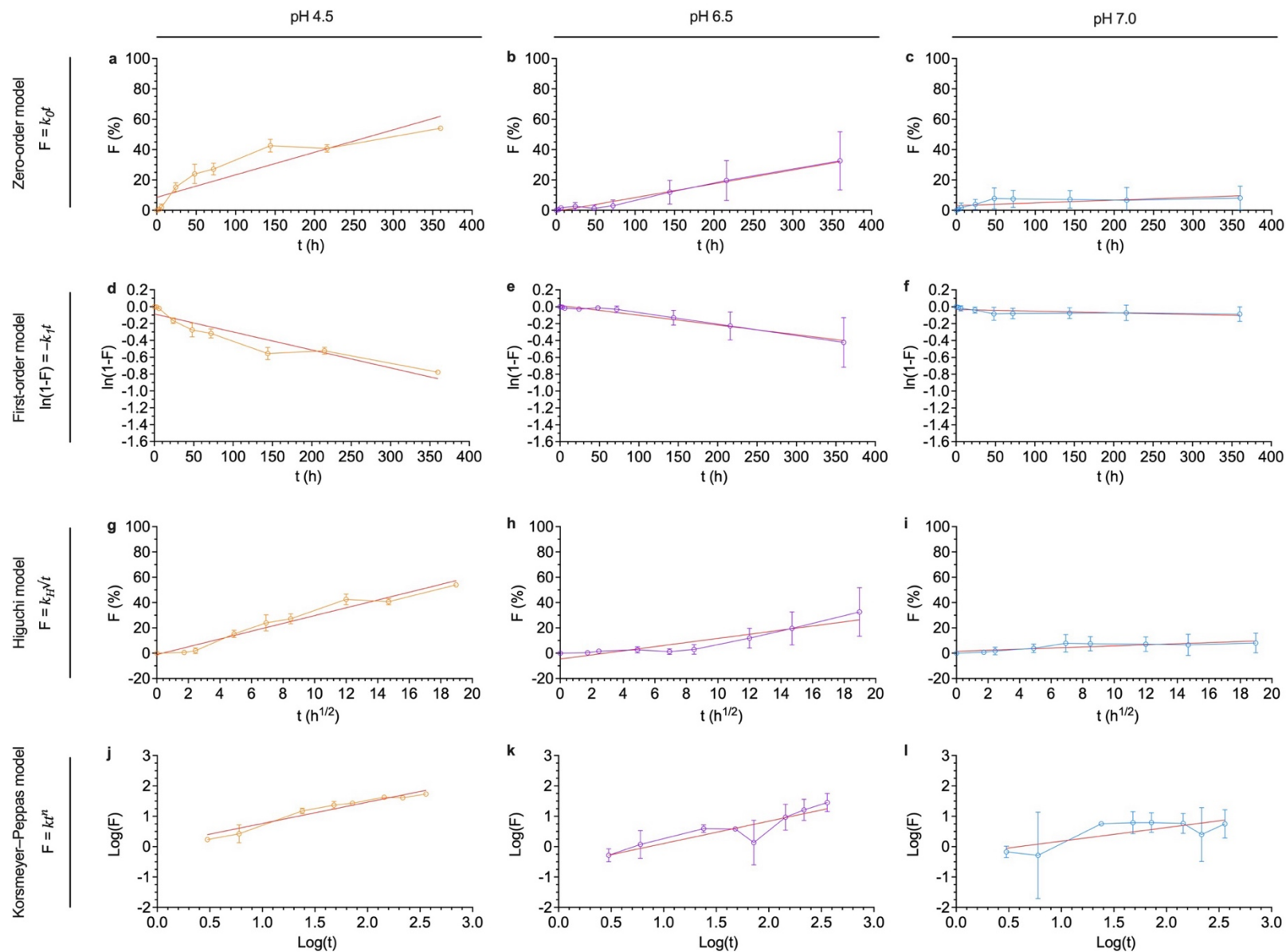


Figure 5.10 Release kinetics of 100 μM doxorubicin-loaded silk nanoparticle (100DOX-SNP) manufactured by the antisolvent precipitation. The drug release profiles were studied at pH 7.0, pH 6.5, and pH 4.5 for 15 days. The kinetic models, including the zero-order model, first-order model, Higuchi model, and Korsmeyer-Peppas model, were used to describe the drug release mechanism. All models were calculated using a linear regression.³³⁰ F indicates the % cumulative drug release up to time t. The k_0 , k_1 , k_H , and k are the kinetic constants obtained from the mathematical models, which indicate the rate of drug release over time. The experiment was conducted as three technical repeats ($n = 3$).



5.3.7. Cytotoxicity

Doxorubicin-loaded silk nanoparticle prepared by the adsorption method was selected as a model for dose-dependent cytotoxicity in MDA-MB-231, DU 145, and RAW 264.7 cells owing to its favourable *in vitro* release behaviour (**Figure 5.9**). The dose–response curve of doxorubicin-loaded silk nanoparticles demonstrated high cytotoxicity comparable to (free doxorubicin), with no significant differences in EC₅₀ values observed between the formulations: 0.569 µg/mL for MDA-MB-231 cells (0.445 µg/mL) (**Figure 5.12a**, **Figure 5.12d**, and **Figure 5.12h**) and 0.057 µg/mL for DU-145 cells (0.067 µg/mL) (**Figure 5.12b**, **Figure 5.12e**, and **Figure 5.12h**). Doxorubicin-loaded silk nanoparticles exhibited lower cytotoxicity than free doxorubicin in RAW 264.7 cells, with a higher EC₅₀ value of 0.857 µg/mL (**Figure 5.12c**, **Figure 5.12f**, and **Figure 5.12h**). Placebo (blank) silk nanoparticles showed no cytotoxicity in all tested cell lines (**Figure 5.12g**).

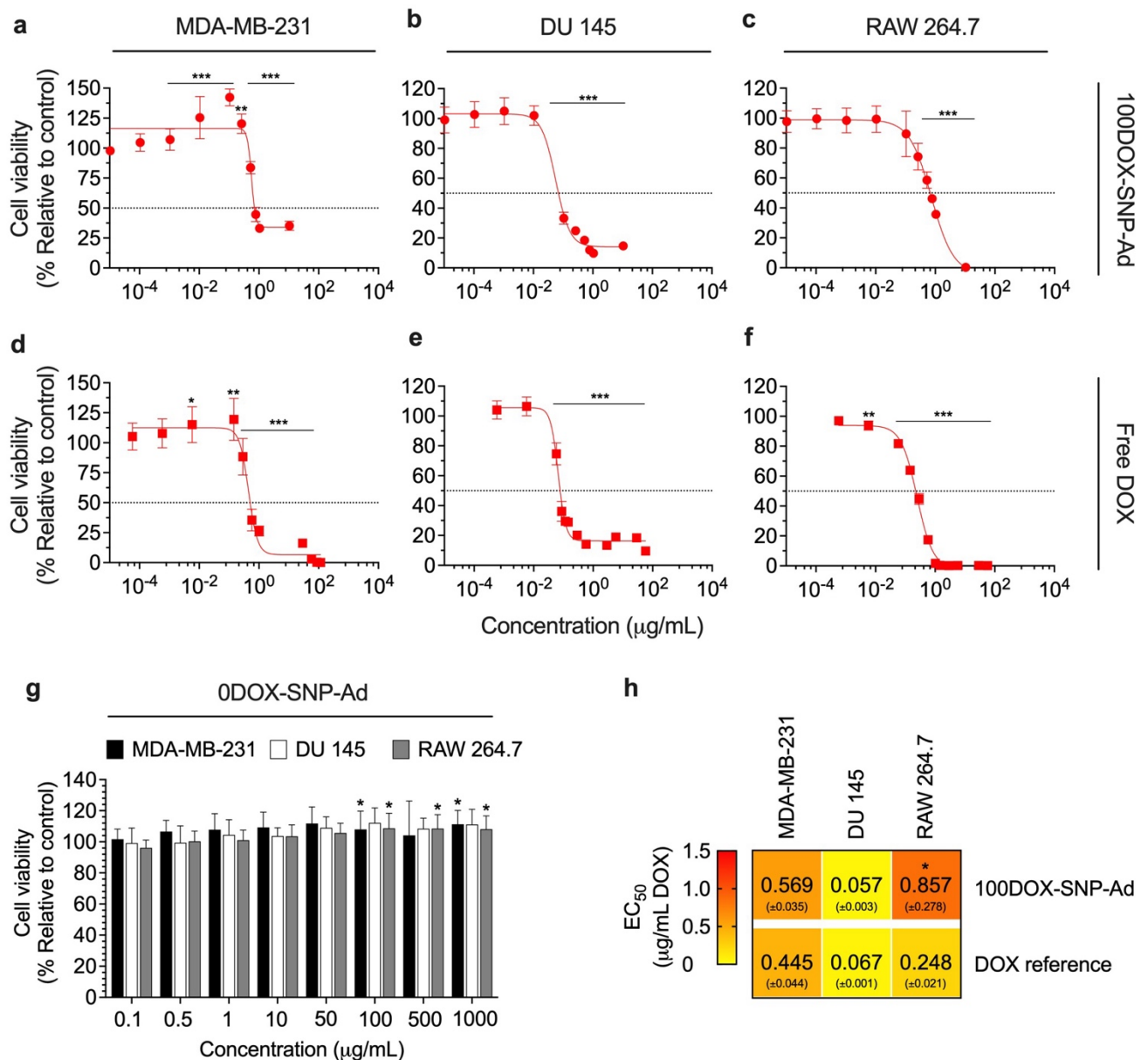


Figure 5.12 Cytotoxicity of doxorubicin-loaded silk nanoparticles. Cells were incubated with doxorubicin-loaded silk nanoparticles (100DOX-SNP-Ad) prepared by the adsorption method for 48 h followed by the CellTiter-Glo assay. Dose-dependent cytotoxicity of **(a-c)** doxorubicin-loaded silk nanoparticles, **(d-f)** free doxorubicin, and **(g)** empty silk nanoparticles. **(h)** The half-maximal effective concentration (EC_{50}) was calculated using the Hill equation. The concentration assignment: μg dried-weight of nanoparticles per mL of dispersant for empty particle; μg of doxorubicin per mL of dispersant for free doxorubicin and doxorubicin-loaded silk nanoparticles. One-way ANOVA and Dunnett's multiple comparisons test were used for statistical analyses: $p < 0.1$ (*), $p < 0.01$ (**), $p < 0.001$ (***). The experiment was conducted as three independent cell experiments with three technical repeats each ($n = 3$). Effects with $p < 0.1$ are reported as exploratory trends only and are not interpreted as statistically significant.

5.3.8. Cell Uptake in MDA-MB-231 Cells

Cellular uptake in MDA-MB-231 cells was evaluated using fixed cell imaging with confocal microscopy. DAPI staining indicated the nuclear position of individual cells and is shown as a blue fluorescence, while the particles were tracked *via* encapsulated doxorubicin and are shown as a green fluorescence. RPMI treatment was incorporated as a control, confirming that no interfering signal was involved in doxorubicin emission range (**Figure 5.13a**). In cells treated with free doxorubicin ($0.5 \times \text{EC}_{50}$), a slight increase in green fluorescence was detected in the nuclear region and the surrounding perinuclear area (**Figure 5.13b**). A dose-dependent response was observed in cells treated with doxorubicin-loaded silk nanoparticles, with MDA-MB-231 cells exhibiting increased uptake at higher treatment doses (**Figure 5.14a-b**).

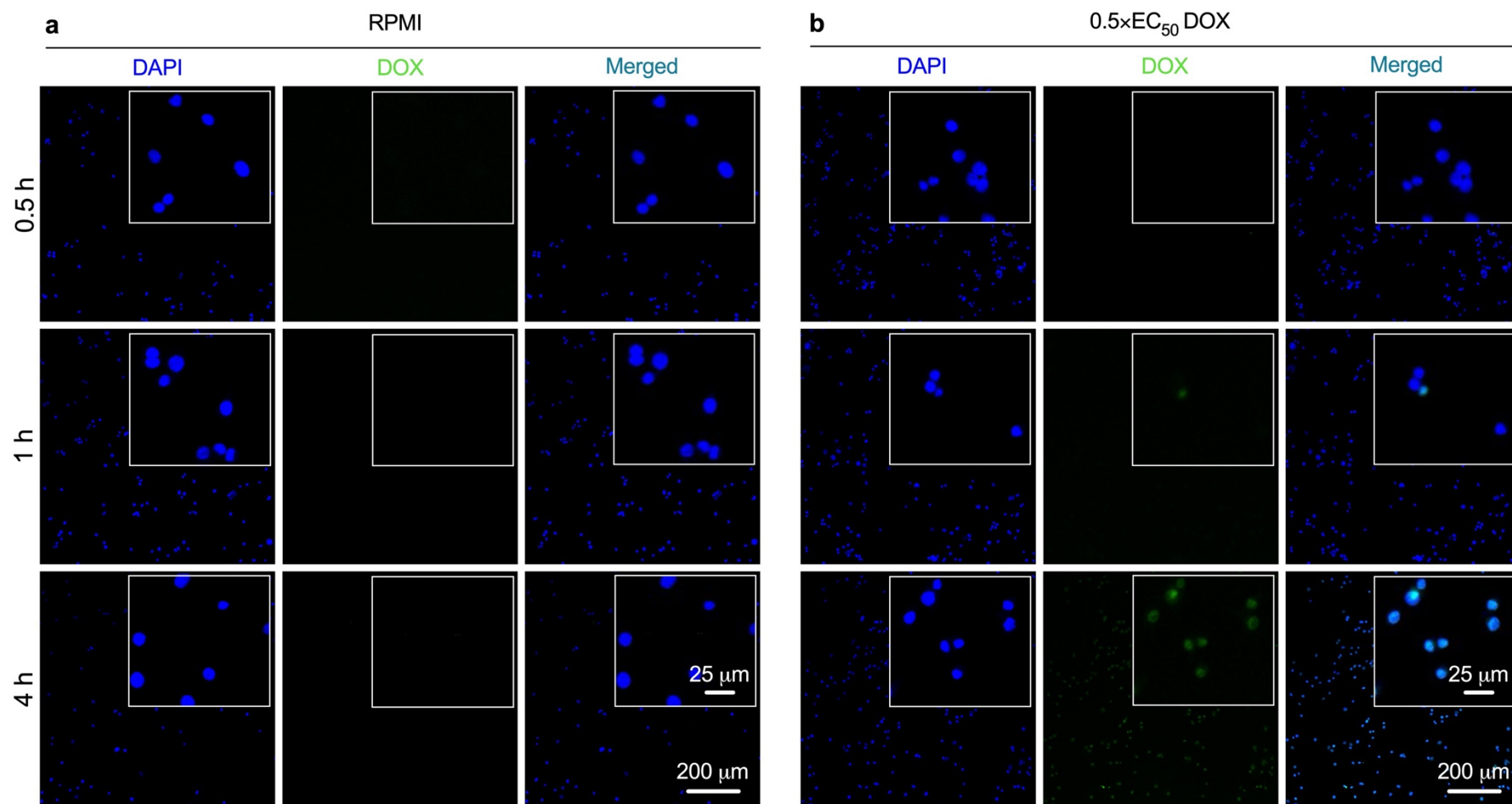


Figure 5.13 Cell uptake study of doxorubicin (DOX) using a fixed cell imaging method. MDA-MB-231 cells were exposed to free DOX at a concentration of $0.5 \times EC_{50}$ for 0.5–4 h. Cells were fixed with 4% paraformaldehyde and stained with 1 $\mu\text{g}/\text{mL}$ DAPI before visualization. The RPMI media served as a negative control. This experiment was conducted as one cell experiment repeat with 3 areas of interest ($n = 1$).

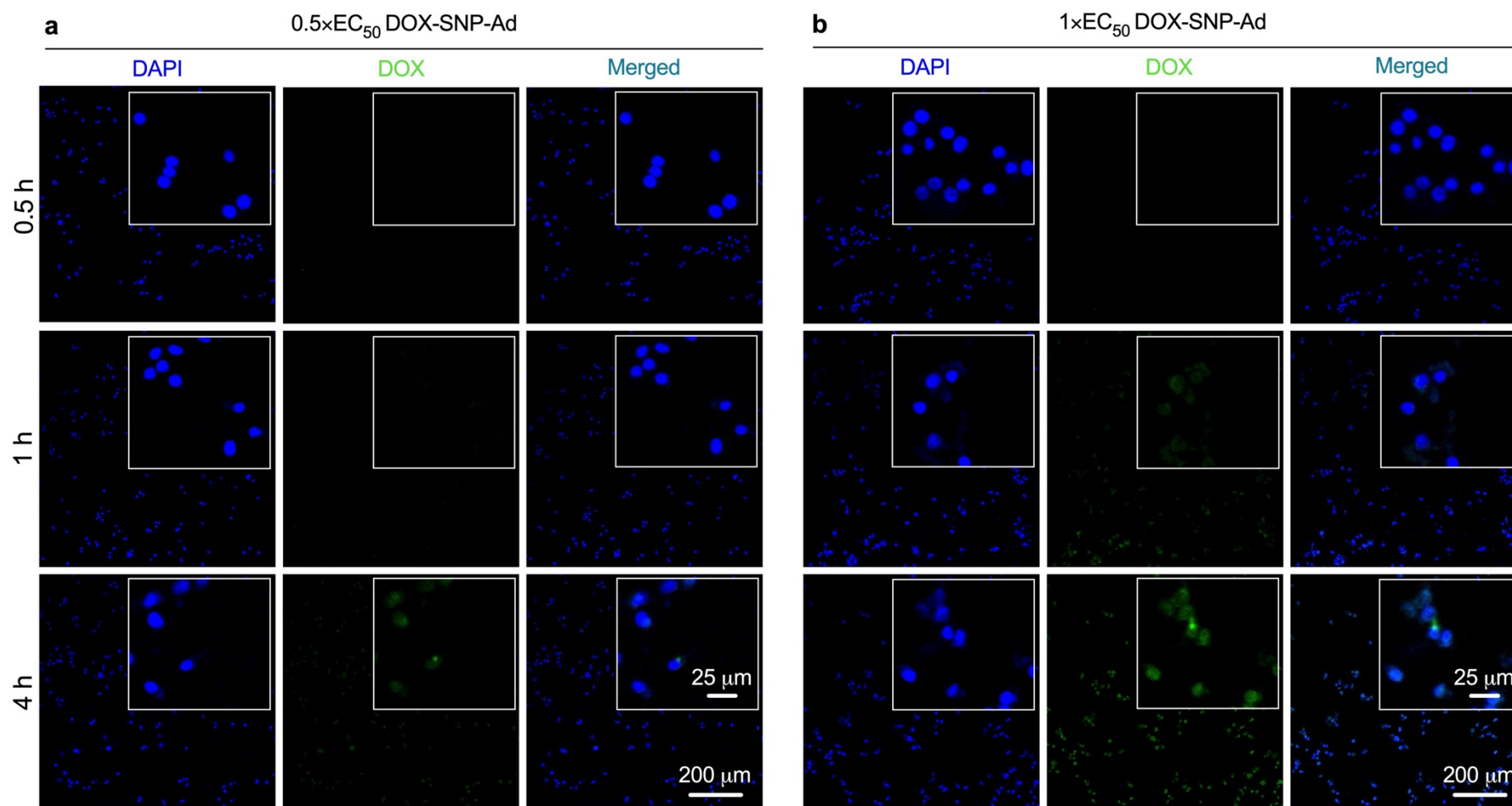


Figure 5.14 Cell uptake study of doxorubicin-loaded silk nanoparticles prepared by adsorption method (DOX-SNP-Ad) using a fixed cell imaging method. MDA-MB-231 cells were exposed to doxorubicin-loaded silk nanoparticles at concentrations of $0.5 \times EC_{50}$ and $1 \times EC_{50}$ for 0.5–4 h. Cells were fixed with 4% paraformaldehyde and stained with $1 \mu g/mL$ DAPI before visualization. This experiment was conducted as one cell experiment repeat with 3 areas of interest ($n = 1$).

5.3.9. Semi-Quantitative and Quantitative Analysis of Intracellular Doxorubicin

To investigate the correlation between doxorubicin and number of cells, the mean fluorescence intensity of both signals was quantified using Fiji/ImageJ software. With longer exposure times (4h), a higher DOX/DAPI ratio was observed in MDA-MB-231 cells. These results demonstrate that the uptake response of MDA-MB-231 cells is both time-dependent and concentration-dependent (**Figure 5.15a**). Furthermore, slight deterioration in nuclear morphology was significantly observed over time, particularly in the doxorubicin-loaded silk nanoparticle treatment groups (**Figure 5.15b**). Quantitative analysis revealed a significant increase in the doxorubicin fluorescence signal, indicating increased uptake in all samples over time. Interestingly, doxorubicin-loaded silk nanoparticles showed significant uptake at 1 h ($1\times\text{EC}_{50}$) and 4 h ($0.5\times\text{EC}_{50}$) (**Figure 5.15c**).

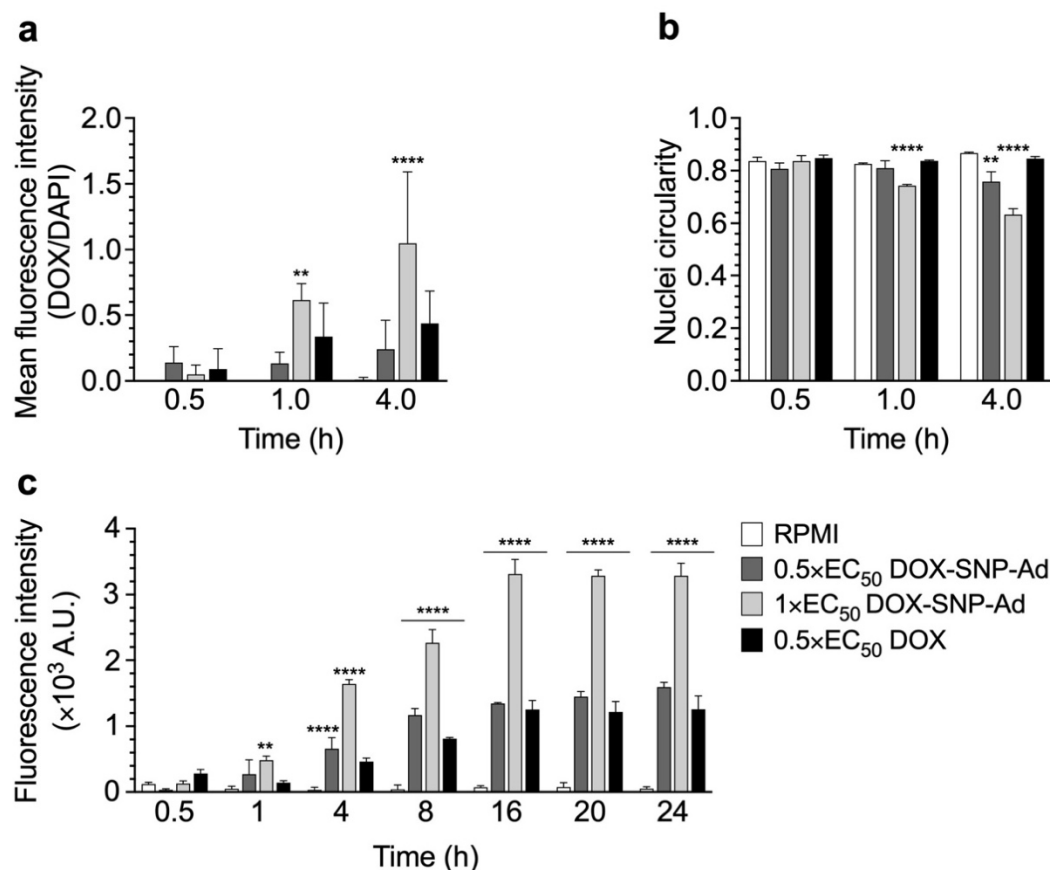


Figure 5.15 Semi-quantitative and quantitative analysis of intracellular doxorubicin studied in MDA-MB-231 cells. Cells were plated in 12-well plate (9375 cells/cm^2) and were exposed to doxorubicin-loaded silk nanoparticles (DOX-SNP-Ad) ($0.5 \times EC_{50}$ and $1 \times EC_{50}$) for 0.5–24 h. RPMI media served as a negative control. Doxorubicin ($0.5 \times EC_{50}$) served as a free drug control. The value of (a) mean fluorescence intensity of DOX/DAPI ratio and (b) nuclei circularity were obtained from confocal images and were computed by Fiji/ImageJ software, facilitating a semi-quantitative analysis for uptake response. (c) The quantitative analysis of intracellular doxorubicin was obtained from lysed cells in 1% (v/v) Triton X-100. The fluorescence intensity was subtracted and expressed in A.U. (Ex/Em= 480/594 nm). Two-way ANOVA and Dunnett's multiple comparisons test were used for statistical analyses: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). The computed DOX/DAPI ratio and nuclei circularity were conducted as one cell experiment repeat with 3 areas of interest ($n = 1$), while quantitative measurement of DOX was conducted as three independent cell experiment repeats ($n = 3$). Effects with $p < 0.1$ are reported as exploratory trends only and are not interpreted as statistically significant.

5.4. Discussion

5.4.1. The Impact of the Manufacturing Methods on Doxorubicin Nanoparticle

Physicochemical Properties

The goal of this chapter was to establish the potential use of silk fibroin precipitation to manufacture silk nanoparticles encapsulating doxorubicin through the incorporation of doxorubicin in the silk fibroin solution during precipitation (DOX-SNP-ASP) and surface adsorption of doxorubicin on silk nanoparticles formed by precipitation (DOX-SNP-Ad).

Significant changes in the physicochemical properties of silk nanoparticles were observed when comparing (1) empty and drug-loaded particles and (2) different encapsulation approaches. Importantly, increased doxorubicin concentration (0–100 μM) considerably increased particle size by the antisolvent precipitation method. The integration of antisolvent nanoprecipitation (principle described in Chapter 2) in the presence of doxorubicin significantly influenced the size-tuning process of silk nanoparticles. This effect may be attributed to doxorubicin altering the degree of nucleation and/or particle growth during particle formation, leading to a significant increase in particle size. The positive ion ($-\text{NH}_3^+$) in doxorubicin enables the interaction with negatively charged residues *via* electrostatic interactions^{331, 332} together with hydrogen bonding,³³² enabling adsorption located near the particle surface, where negatively charged residues are located. Another working hypothesis is that the aromatic ring system in doxorubicin intercalates with aromatic amino acid residues in the core of a β -sheet-rich particle (**Figure 5.2a** and **Figure 5.2c**).¹⁵⁵

Doxorubicin silk nanoparticles prepared by the adsorption method did not exhibit any significant changes in particle size, except for the highest doxorubicin concentration (100 μM) (**Figure 5.2d**). With the changes in ZP value as doxorubicin increased—a sharp decline in antisolvent precipitation formula and a gradual decline in the adsorption formulation—it is hypothesized that doxorubicin directly influences the particle surface charge toward less negative

value; however, the ZP of doxorubicin-loaded silk nanoparticles remains negative, identified as a stable nanoparticle, as its ZP was in between -13 to $+13$ mV (**Figure 5.2c**).³³³ Even though an increased variability in PDI values was observed in the adsorption method; however, it was still highly homogeneous (PDI <0.2) (**Figure 5.2b**).³³³

FE-SEM images confirmed that doxorubicin encapsulation and the manufacturing method did not significantly impact silk nanoparticle morphology, retaining a circular morphology (circularity value ~ 1) (**Figure 5.3a-b**).²⁶¹ Taken together, doxorubicin encapsulation can induce changes in silk nanoparticle formulation physicochemical attributes and stability compared to previously reported characteristics.³³⁴ Therefore, the functionality of doxorubicin-loaded silk nanoparticles should be carefully considered.

5.4.2. The Impact of Manufacturing Methods on Protein Secondary Structure Content

Encapsulation of drug cargo within a polymeric particle and/or on its surface may disrupt the overall particle hierarchical structure, affecting its mechanical stability and release behaviour.³³⁵ FTIR was performed to investigate protein secondary structure of silk nanoparticles to determine if higher doxorubicin concentration and manufacturing method enhance silk structural changes (**Figure 5.4** and **Figure 5.5**). The antisolvent precipitation method resulted in a higher β -turn content and a lower random coil content in comparison to the adsorption method (**Figure 5.5a**). A possible explanation is that doxorubicin encapsulation by adsorption under agitation (18 hours) perturbs the native folding of the silk chains. This effect is absent in placebo silk nanoparticle formulations. This could trigger silk structural changes,^{90, 336} leading to the loss of β -turns and an increase in β -sheet and random coil content (**Figure 5.5a**).

Nevertheless, both manufacturing methods produced β -sheet-rich silk nanoparticles (**Figure 5.4** and **Figure 5.5**), which is advantageous for retaining integrity and stability^{249, 337} and play important roles in controlling drug entrapment,^{249, 337} drug release,^{249, 337} and degradation performance.^{48, 49, 338}

5.4.3. The Impact of Doxorubicin Encapsulation on Nanoparticle Physicochemical Attributes and Drug Encapsulation Studied with Frit-Inlet Asymmetric Flow Field-Flow Fractionation.

FI-AF4 was used to comprehensively investigate doxorubicin-loaded silk nanoparticle formulation properties.^{150, 339} Silk nanoparticle tracking and size analysis were performed using the MALS and UV detectors, while doxorubicin was monitored by fluorescence detection. Doxorubicin-loaded silk nanoparticles manufactured by antisolvent precipitation had broader fractogram traces and longer retention times than the adsorption formulation, indicating a broader size distribution and larger particle size (**Figure 5.6** and **Table 5.2**). This agrees with the DLS results that doxorubicin encapsulation increased hydrodynamic diameter (D_H) (**Figure 5.2a**).

Principally, R_g indicates the conformational folding and compactness of proteins or nanoparticles,^{340, 341} where a smaller R_g reflects a more compact or folded structure, whereas a larger R_g reflects a more swollen, extended, or unfolded structure.³⁴² Together with the alteration in protein secondary structure (**Figure 5.5**), these findings imply that the overall particle structure was affected. It has been suggested that attractive van der Waals forces exist between particles and the membrane surface, correlating with nanoparticle core density. Higher core density nanoparticles exhibited longer retention times than lower (bulk) density nanoparticles, resulting in a size-independent influence on separation.³⁴³ It is hypothesized that co-precipitation with doxorubicin using the antisolvent precipitation offers higher core density (compacted) nanoparticles, leading to longer travel time in the FI-AF4 channel. However, the adsorption method enables a stronger retention of the morphology of doxorubicin-loaded particles during separation (**Figure 5.6** and **Table 5.2**)

As anticancer nanocarriers, nanoparticles circulate in the bloodstream to promote tumour accumulation while reducing toxicity to healthy tissue. Their physicochemical properties—including size, surface charge, and morphology—are critical determinants of tumour

infiltration and biodistribution.¹⁶⁰ Sizes of 10–200 nm prolong circulation and reduce uptake by the reticuloendothelial system, while surface properties and morphology govern biological interactions and cellular uptake, with spherical particles offering optimal circulation.^{161, 164} Overall, FI-AF4 analysis demonstrates that silk nanoparticles show strong potential as anticancer drug carriers, maintaining size and shape under high-speed flow and effectively protecting payloads by minimizing drug loss and improving recovery (**Table 5.2**).

5.4.4. The Composition and Particle Homogeneity of Doxorubicin-Loaded Silk Nanoparticles (Adsorption Formulation) Studied by Single Particle Automated Trapping Analysis (SPARTA)

Raman spectroscopy is a label-free technique that provides Emolecular fingerprints via inelastic laser scattering, enabling diverse sample analyses.³²¹ Single Particle Automated Trapping Analysis (SPARTA) integrates Raman spectroscopy with optical trapping to analyse individual particles without substrate interference or labelling, offering high-throughput compositional assessment.³²¹ SPARTA has gained increasing interest in pharmaceutical research, including studies of extracellular vesicles as cancer biomarkers and evaluations of particle composition, homogeneity, subpopulations, and drug loading location.³⁴⁴⁻³⁴⁶

The protein characteristic of silk nanoparticles was obtained as illustrated in the 421 to 1800 cm^{-1} Raman shift, indicating a strong protein characteristic: amide stretches, and phenylalanine. In contrast, doxorubicin exhibited characteristic signals, which were observed to be slightly shifted compared to previously reported values (**Figure 5.7a**, **Table 5.3**, and **Table 5.4**).^{326, 327} One possible explanation is that the excitation energy, molecular form, and chemical environment of the doxorubicin molecule can influence its Raman shift characteristics, as reported elsewhere.³²⁷ Moreover, despite the overlap between doxorubicin and protein peaks, a significant increase in signals assigned to doxorubicin was observed. This result demonstrates a clear distinction between the drug-loaded and empty particle subpopulations—indicating high

homogeneity—particularly evident in the univariate analysis of each crucial doxorubicin peak, especially at 1209 cm^{-1} (**Figure 5.7b–h**).³²⁶ In this case, bivariate analysis was used to examine correlation patterns—comparing empty particles with doxorubicin-loaded particles—across two signal peaks. The results confirmed a consistent increase in doxorubicin signals, observed across multiple peaks throughout the spectrum (**Figure 5.8a–u**).

On the other hand, a notable reduction in signal was observed at 1660, 850, and 825 cm^{-1} . It is proposed that loading doxorubicin into the particles affects silk fibroin structure, leading to alterations in the Raman intensity. Since these peaks correspond to the Amide I ($\text{C}=\text{O}$) stretch at 1660 cm^{-1} and tyrosine residues at 850 and 825 cm^{-1} ,³²⁸ it was hypothesized that doxorubicin may interact with silk fibroin, potentially forming hydrogen bonds *via* its NH_3^+ group with the $\text{C}=\text{O}$ group of protein,³³² as well as hydrophobic interactions with the tyrosine residues. These interactions alter the protein's microchemical environment and structure, leading to an apparent signal reduction that indicates the protein-DOX complex (**Figure 5.7a**).³⁴⁷

In conclusion, this study demonstrates that SPARTA is compatible with silk nanoparticles, enabling high-throughput, reliable drug-particle composition analysis without labelling or modification. Given its potential to extract multiplexed information—such as composition, drug content, and particle size—SPARTA warrants further investigation.³²¹ Future studies should incorporate bivariate analyses including variables such as particle size, drug loading, and drug type to better understand inter-variable relationships and support the optimization of silk-based drug-delivery systems.

5.4.5. The Impact of Drug Encapsulation Methods on Encapsulation Efficiency

Because the antisolvent precipitation and adsorption methods are based on different fundamental principles, they may yield different outcomes for drug encapsulation and loading. The results support the conclusion that silk nanoparticles exhibit remarkable drug loading capacity. As the

concentration of doxorubicin (0–100 μ M) increased, the drug encapsulation efficiency increased for both methods (**Table 5.5**).

Drug encapsulation via the adsorption method relies on the particle's physicochemical properties influencing drug adsorption capacity; for example, particle size,³⁴⁸⁻³⁵⁰ morphology,³⁴⁸⁻³⁵⁰ surface area,^{348, 349} pore architecture (e.g., diameter, volume, structure),^{348, 351} and the chemical environment of the particle surface (e.g., functional groups, charge properties, etc.).^{332, 350} In comparison, the antisolvent precipitation enables the silk to assemble, forming a β -sheet-rich particle core.²³ It can be speculated that as the β -sheet formation occurs, doxorubicin can be more stabilized with the β -sheet network due to its aromatic moiety,^{249, 352} leading to drug entrapment in a way that simultaneously promotes particle growth through a condensation—refers to the adsorption of the free silk chains onto the particle surface, enlarging the particle size after nucleation, and/or an agglomeration of particle—involves in particle collision frequency/efficiency and particle-particle interactions (e.g., van der Waals force and electrostatic repulsion).⁷⁵ The antisolvent precipitation also allows the drug absorption on the particle surface, as the particle number increases in the batch reaction.

The drug loading content was additionally determined using fluorescence detection alongside UV–Vis spectroscopy to strengthen the reliability of the measurements, as each method has its own inherent limitations. UV–Vis spectroscopy provides an indirect estimation of drug content based on the amount of drug remaining in the reaction batch, whereas fluorescence detection via FI-AF4 directly measures the drug entrapped in the silk nanoparticles. Therefore, variations in drug content measurements may be observed in this study. According to the fluorescence result, the manufacturing method had no significant impact on drug loading content; however, it plays an important role in controlling drug diffusion, as a significant difference in drug loss occurs (**Table 5.5**). Furthermore, SPARTA analysis confirmed the exceptional homogeneity of the drug-loaded nanoparticles, with ~95% of the population exhibiting consistent

drug incorporation, highlighting their uniformity and potential for predictable therapeutic performance (**Figure 5.7**, **Figure 5.8**, and **Table 5.5**).

5.4.6. The Release Behaviour of Doxorubicin-Loaded Silk Nanoparticles

Drug release studies are essential to evaluate formulation-dependent dissolution behaviour,^{353, 354} determine release rates and durations required for therapeutic efficacy and *in vivo* safety while reducing animal studies,³⁵⁵ and assess the suitability of selected polymers and manufacturing methods for optimizing drug-delivery performance and therapeutic outcomes.^{354, 356, 357}

This thesis suggested that the silk nanoparticle is a pH-responsive nanocarrier, exhibiting a higher doxorubicin release at lower pH ($\text{pH } 7.0 < \text{pH } 6.5 < \text{pH } 4.5$) (**Figure 5.9a-b**). One possible implications is that with a pI value of 4.39 for silk H-chains,³⁵⁸ it is hypothesized that increased protonation of the NH_2 groups may occur at lower pH, facilitating the changes in protein interactions,^{359, 360} protein folding and assembling characteristics, thereby increasing chain flexibility, loosening particle structure, and weakening drug-protein interactions, which led to enhanced drug release at low pH.³⁶¹

The four comparative kinetic models were applied to characterize the multi-mechanistic release from silk nanoparticles (**Figure 5.10** and **Figure 5.11**). The R-squared (R^2) reflects model fit; higher R^2 values indicate a better fit.³²⁹ The rate constant (k) quantifies the drug-release rate over time.³³⁰ The results show that the Higuchi and Korsmeyer–Peppas models most accurately describe doxorubicin release from silk nanoparticles at acidic pH. Moreover, the release behavior was consistent with the FI-AF4 study, demonstrating higher drug release/loss in the adsorption-based formulation during separation (**Figure 5.9** and **Table 5.3**)

Due to the water solubility of doxorubicin, its release from silk nanoparticles followed diffusion-controlled kinetics, as described by the Higuchi model.^{322, 323, 362, 363} The release profiles were also well fitted by the Korsmeyer–Peppas model, where the exponent (n) indicates the release mechanism.³²² At pH 4.5, most formulations exhibited anomalous (non-Fickian) diffusion

(**Figure 5.9c**), suggesting that silk chain relaxation governs doxorubicin diffusion.^{364, 365} The higher release rate observed for adsorption-based formulations is attributed to relaxation-induced diffusion of more mobile surface silk chains, whereas encapsulated drug is retained within less flexible, β -sheet-rich regions, resulting in slower diffusion and reduced release rates.³⁶⁴ However, I proposed that release kinetics should be further elucidated, as the dialysis method may restrict diffusion, leading to an underestimation of the true release rate. In addition, sink conditions may not be maintained, while drug adsorption and membrane clogging can further affect accuracy and reproducibility.

5.4.7. Cytotoxicity Effect of Doxorubicin-Loaded Silk Nanoparticles and Cell Uptake

With the increasing use of nanoparticles as drug nanocarriers, cytotoxicity has become a key factor in their evaluation.^{366, 367} *In vitro* cell culture models are widely used as first-line screening tools due to their low cost, ethical acceptability, and reproducibility, and are particularly valuable for assessing the cytotoxicity of silk nanoparticles and associated drugs.^{366, 368, 369} The dose-response curve of doxorubicin-loaded silk nanoparticles showed cytotoxicity comparable to free doxorubicin, with a similar, slightly left-shifted profile indicating comparable potency. Empty silk nanoparticles were non-cytotoxic, confirming that the observed effects were due to encapsulated doxorubicin. Notably, reduced cytotoxicity was observed in RAW 264.7 immune cells, suggesting preserved drug efficacy with improved selectivity toward cancer cells (**Figure 5.12**).

MDA-MB-231, a triple-negative breast cancer (TNBC) cell line, is highly aggressive, rapidly proliferating, and is an ideal model for studying cytotoxicity, nanoparticle uptake, and drug delivery efficiency;^{370, 371} thereby MDA-MB-231 was selected as the first study model in this thesis. An increasing fluorescence intensity of the DOX/DAPI ratio over time suggests that doxorubicin accumulation in MDA-MB-231 cells occurs progressively, indicating effective intracellular uptake and retention. The localization of doxorubicin near the nuclear region aligns

with its mechanism of action, as it can intercalate with DNA and exert cytotoxic effects within the nucleus,¹⁵⁵ which may contribute to disruptions in nuclear morphology (**Figure 5.13**, **Figure 5.14**, and **Figure 5.15b**).^{372, 373} Notably, the increased uptake observed with doxorubicin-loaded silk nanoparticles at earlier time points suggests that this nanoparticle system may enhance cellular internalization and drug transport. Furthermore, higher uptake at 1×EC50 compared with 0.5×EC50 supports a dose-dependent internalization mechanism, indicating that the level of cellular exposure influences nanoparticle delivery efficiency (**Figure 5.13**, **Figure 5.14**, and **Figure 5.15**).

However, further studies on the uptake mechanism are warranted, as understanding this process could potentially enhance the functionality of silk nanoparticles and maximize their therapeutic efficiency in triple-negative breast cancer cells. Furthermore, limitations of using RAW 264.7 murine macrophages should be considered more, as its phagocytic activity may differ from primary macrophages or human macrophages, influencing nanoparticle internalization and toxicity results. The endotoxin contamination level of silk nanoparticles should also be assessed, as endotoxin contamination can hinder the translation of nanoparticles for biomedical applications.

5.5. Conclusion

This chapter demonstrates how the physicochemical properties of silk nanoparticle formulations are shaped by two distinct drug encapsulation methods. Using a comprehensive analytical pipeline, particularly FI-AF4 and SPARTA, the study established a preliminary understanding of how encapsulation impacts the physicochemical properties of silk nanoparticles and their pH-responsive payload release. The adsorption-based formulation enhanced the *in vitro* release rate while demonstrating potential selectivity toward cancer cells. Together, these findings support the rational design of tunable silk-based nanocarrier systems for targeted doxorubicin delivery.

Chapter 6 General Discussion and Future Directions

6.1. General Overview

Bombyx mori silkworm silk has received considerable attention as a promising biomedical material, as evidenced by its broad-spectrum applications, ranging from medical tensile¹ to nanomedicine (reviewed in²). Apart from silk tensile strength and toughness,⁸ biocompatibility,³⁻⁵ and biodegradability,⁶ the impressive silk fiber production process of the silkworm—at ambient temperature—inspires us with various key factors that could be useful for *ex vivo* silk material processing,²⁶ including the significant reports of Ca^{2+} and K^{+} in modulating silk conformational transition.^{26, 28, 31}

This thesis followed the silk nanoparticle manufacturing pipeline as described elsewhere²³ incorporating the previously uncharacterized impacts of bioinspired counter ions (Ca^{2+} and K^{+}) in *B. mori* silk nanoparticles generated by the antisolvent precipitation method. The study aims and research gaps were highlighted as follows.

(1) The primary objective of this thesis is to fabricate silk nanoparticles with enhanced physicochemical properties and improved stability. Metal ions are hypothesized to act as key modulators of silk fibroin structural alteration and conformational transitions. Based on this premise, this research explores the use of ions as a beneficial factor for enhancing silk nanoparticle formation during synthesis through structural regulation and modulation of physicochemical properties. This thesis demonstrates that ions play an essential role in fine-tuning silk nanoparticle characteristics, particularly by governing particle size, surface charge, and the silk assembly mechanisms during nanoparticle synthesis. Collectively, these effects result in both improved nanoparticle quality and increased production yield. Although changes in nanoparticle properties have been observed, colloidal stability has not yet been assessed. This is crucial, as it directly affects dispersion, shelf-life, and drug delivery performance (Chapters 2 and 3).^{245, 278}

(2) As ions effectively modulate silk nanoparticle properties, particularly particle size and surface charge, colloidal stability is examined in this chapter. The presence of ions is expected to influence particle-particle interactions by altering electrostatic forces, thereby either enhancing or reducing stability. This chapter addresses this question using a combination of orthogonal analytical techniques, including DLS, ELS, NTA, FE-SEM, AF4, and EAF4, with the additional aim of establishing a robust analytical pipeline for silk nanoparticle characterization. The results demonstrate that ions play a crucial role in balancing electrostatic repulsion and attraction, thereby enhancing colloidal stability even under stress conditions such as acidic pH and elevated temperature. These findings highlight the advantages of silk fibroin as a nanocarrier with a low risk of agglomeration; however, its downstream drug loading and release ability remain to be investigated (Chapter 4).³³⁴

(3) The promising physicochemical properties of silk nanoparticles motivated a detailed evaluation of their drug loading and release behaviour. Their responsiveness to acidic pH, combined with intrinsic protein characteristics, indicates inherent pH-sensitive behaviour, which is highly advantageous for controlled anticancer drug delivery. Distinct loading strategies, such as antisolvent precipitation and adsorption, were found to markedly influence drug loading efficiency and release kinetics. Silk nanoparticles without ions were employed as a representative system to first establish biomaterial compatibility. The results confirm that silk nanoparticles are effectively pH-responsive, with adsorption emerging as the superior method to achieve controlled drug release and enhanced cytotoxic efficacy of doxorubicin-loaded nanoparticles against cancer cells. However, the detailed mechanisms of cellular uptake and cytotoxic action, as well as the influence of ions on these properties, have not yet been explored. Therefore, these aspects should be highlighted as key research gaps for future studies (Chapter 5).

6.2. Future Directions

6.2.1. Drug Loading and Controlled Drug Release of Silk Nanoparticles

Chapter 5 demonstrated the development of silk nanoparticle formulations with varying doxorubicin concentrations (50–100 μM), showing $\sim 65\%$ encapsulation efficiency in silk nanoparticles. The drug loading efficiency was influenced by the encapsulation methods and the counterions. Even though this thesis successfully formulated doxorubicin-loaded silk nanoparticles with favourable characteristics, the drug loading content of $\sim 1\text{--}4\%$ w/w should be increased to over 10% w/w in silk nanoparticles, as it could potentially improve their therapeutic application.^{297, 374} Furthermore, studying the drug loading capacity could inform us about the maximum amount of drug that can be incorporated while preserving the essential physicochemical properties of silk nanoparticles as nanocarriers, as well as determining the minimal biomaterial requirement for each drug. Further studies on controlled, sustained, and specific drug release to cells and/or tissues are needed, with a greater focus on therapeutic activity, stealth properties, and systemic circulation time to ensure that the system meets the required standards for tumour-targeted applications.³⁷⁵⁻³⁷⁷

6.2.2. Biodegradation of Silk Nanoparticles

Ideally, biodegradable biomaterials should degrade safely after fulfilling their function, have a shelf-life aligned with the anticipated therapeutic duration of the disease, and degrade *via* mechanisms that are compatible with tissue regeneration.³⁷⁸ Silk biodegradation depends on silk molecular weight and structure: lower molecular weight and less compact structures enhance enzymatic hydrolysis,⁴⁵ with a higher β -sheet content exhibiting slower degradation.⁴⁹ This suggests that the biodegradation rate of silk biomaterials can be regulated through control of silk fibroin secondary structure.

The superior physicochemical stability of Ca^{2+} - and K^{+} -mediated silk nanoparticle formulations was validated when compared with the control silk nanoparticles (Chapter 4). These nanoparticles exhibited favourable colloidal stability and excellent retention of particle size and morphology over 56 days of storage. However, due to their β -sheet-rich (~60%)—a structural feature that influences enzymatic degradation^{48, 49}—the biodegradability of the resulting nanoparticles should be carefully considered. The *in vitro* degradation of silk nanoparticles using various proteolytic enzymes (e.g., protease XIV, α -chymotrypsin, papain, and lysosomal enzymes) should be further investigated, as these systems have been widely used as models in previous work to study silk nanoparticle degradation.^{6, 7, 379, 380}

6.2.3. Biocompatibility

Silk nanoparticles are well-known and widely developed drug nanocarriers in drug delivery research, and their biocompatibility and safety have been extensively evaluated.^{3, 5, 381} From a nanomedicine perspective, substantial evidence on the hemocompatibility of silk has emerged, highlighting it as a critical factor in nanoparticle design for therapeutic applications.^{5, 382}

In this thesis, the high biocompatibility of empty silk nanoparticles was assessed *in vitro* (Chapters 2–3), along with the favorable efficacy observed with doxorubicin-loaded silk nanoparticles against cancer cell lines (Chapter 5). However, further key considerations—such as circulation time, effects on coagulation, hemolysis, inflammation, and clearance by circulating immune cells or the reticuloendothelial system of the liver, spleen, and bone marrow—should be investigated to reinforce the systemic safety of silk nanoparticles and support their use in targeted drug delivery.³⁸² Blood compatibility and protein corona formation are critical factors influencing the safety and performance of nanomedicines. Future work could focus on strategies to reduce serum protein adsorption on silk nanoparticles, thereby minimizing or modifying the biomolecular corona. Improving control over the corona may enhance blood compatibility, particle stability, cellular uptake, and overall therapeutic efficacy.^{382, 383} Future work should also

evaluate how the physicochemical properties of silk nanoparticles affect biocompatibility and immunotoxicity, as accurate characterization combined with toxicological assessment is essential to optimize design, predict *in vivo* behavior, and enhance therapeutic efficacy.³⁸⁴

6.2.4. Cellular Uptake of Silk Nanoparticles

This thesis highlighted the potential of silk nanoparticles as a lysosomotropic nanomedicine, responsive to low-pH environments, with indications that the efficacy of doxorubicin is largely preserved (Chapter 5). However, the mechanism of action remains unproven. As a promising intracellular drug delivery, silk nanoparticles are taken up into cells *via* endocytosis and subsequently trafficked to lysosomes, as reviewed elsewhere.^{2, 131} Therefore, designs for lysosomotropic drug delivery are required, in which the silk nanoparticles respond to the low pH environment and lysosomal enzymes.²⁹⁷ The degradation of silk nanoparticles by mammalian lysosomal enzymes was shown to mimic an ideal environment for lysosomes.⁶ Collectively, future study on cellular uptake mechanism and intracellular trafficking pathways would provide additional mechanistic and subcellular co-localization information, supporting the design of drug delivery systems and informing target biodegradation profiles.

In conclusion, this thesis demonstrates that (1) Ca^{2+} and K^{+} ions modulate silk nanoparticle formation, affecting production yield, and physicochemical properties—particularly particle size. (2) Stability was found to be condition-dependent, with significant limitations in physiologically relevant salt environments. (3) A prototype system demonstrated that silk nanoparticles can be loaded with doxorubicin and exhibit pH-responsive release; however, this has not yet been fully validated for ion-mediated formulations. Therefore, future work will explore additional ion-mediated designs and *in vitro* and *in vivo* evaluation to enhance targeted delivery and therapeutic efficacy. These findings provide a foundation for developing tunable silk-based nanocarriers for clinical applications.

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Appendix A-F

Appendix A

Abstract accepted for poster presentation at SIPBS Research Day 10 October 2025, Strathclyde Institute of Pharmacy and Biomedical Science, University of Strathclyde, Glasgow, United Kingdom.

Modulation of Silk Nanoparticle Physicochemical Properties Through Calcium Ion Incorporation

Roamcharern, N.; Punnabhum, P.; Seib, F. P.; Rattray, Z.

Biomaterials and synthetic polymers are extensively utilized in biomedical applications due to their advantageous biological and physical traits, such as biocompatibility, biodegradability, and payload stability. Among these biomaterials, silk fibroin (SF) has drawn attention as a viable option for drug delivery, largely because of its adaptable secondary structure. Therefore, the role of calcium ions (Ca^{2+}) in modulating the conformation of silk may offer the potential for improving the manufacture of silk nanoparticles (SNPs) at both laboratory and industrial scales. In this investigation, SNPs were produced via antisolvent precipitation in a semi-batch in the presence of Ca^{2+} , and their physical and chemical characteristics were investigated using dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), electrophoretic light scattering (ELS), and electric asymmetric flow field-flow fractionation (EAF4-UV-MALS-DLS). The particle diameter determined by DLS aligned with NTA results, averaging around 100 nm. Notably, Ca^{2+} had an insignificant effect on the polydispersity index (PDI), although it did influence the zeta potential, which was approximately -40 mV. The study also highlights a preliminary separation profile of SNPs based on size-dependent diffusion, showing how Ca^{2+} affects size distribution and agglomeration behaviour. In summary, incorporating Ca^{2+} during SNP production in a semi-batch format significantly influenced nanoparticle size control and zeta potential, suggesting its utility as a way of fine-tuning SNP characteristics during manufacturing.

Appendix B

Abstract accepted for oral presentation at **16th APS PharmSci 2025** 9–11 September 2025, Cardiff, United Kingdom.

Ion-Mediated Modulation of Doxorubicin Encapsulation and Physicochemical Properties of Silk Nanoparticles: Role of Calcium and Potassium Ions Interaction

Roamcharern, N.; Seib, F. P.; Rattray, Z.

Nanomedicine can reduce chemotherapy side effects by optimizing drug distribution through physicochemical design. Bioinspired systems improve anticancer drug efficiency, bioavailability, and pharmacokinetics. Recently, Silk fibroin has emerged as a protein-based nanocarrier due to its biocompatibility, biodegradability, and processability. In this study, doxorubicin-loaded silk nanoparticles (DOX-SNPs) were prepared via antisolvent precipitation, with Ca^{2+} added to promote SF assembly and drug encapsulation. The incorporation of Ca^{2+} and DOX slightly increased the silk nanoparticles diameter with increasing DOX concentration, with measured sizes ranging from 110 to 133 nm. DOX had no significant impact on the polydispersity index, which remained around 0.1, while the zeta potential was slightly altered, becoming less negative, from approximately -35 to -28 mV. Moreover, Ca^{2+} significantly enhanced the encapsulation efficiency at all DOX concentrations, reaching 86–91% compared to 46–76% without Ca^{2+} . This highlights the potential of counterions in optimizing silk nanoparticle properties for drug delivery.

Appendix C

Abstract accepted for poster presentation at **Samaggi Academic Conference and Careers Fair (SACC) 2025** 1 February 2025, London, United Kingdom.

Modulation of Silk Nanoparticle Physicochemical Properties through Calcium Ion Incorporation

Roamcharern, N.; Punnabhum, P.; Seib, F. P.; Rattray, Z.

Biomaterials and synthetic polymers are widely used in biomedical applications due to their beneficial biological and physical properties, including biodegradability, biocompatibility, and payload stability. Silk fibroin (SF) has emerged as a promising candidate for drug delivery due to its favourable characteristics, which are governed by its tunable secondary structure. Therefore, the role of calcium ions (Ca^{2+}) in modulating the conformation of silk may offer the potential for improving the manufacture of silk nanoparticles (SNPs) at both laboratory and industrial scales. In this study, SNPs were synthesised using nanoprecipitation in a semi-batch format in the presence of Ca^{2+} , and their physicochemical properties were analysed using dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), electrophoretic light scattering (ELS), and electric asymmetric flow field-flow fractionation (EAF4-MALS-UV-DLS). The particle size measured by DLS was consistent with NTA measurements, with a reported diameter of approximately 100 nanometres. Interestingly, the presence of Ca^{2+} had minimal impact on the polydispersity index (PDI); however, the zeta potential ranged from approximately -40 mV. The study also provides an initial separation profile of the SNPs, based on size (diffusion), revealing the influence of Ca^{2+} on SNP size distribution and agglomeration behaviour. In conclusion, the Ca^{2+} inclusion during SNP formulation had a great impact on SNP manufacture in the semi-batch format, enabling control of nanoparticle size and altering measured zeta potential. These findings can inform the use of Ca^{2+} as a strategy for modulating SNP physicochemical properties during manufacturing, providing SNPs with optimal characteristics.

Appendix D

Abstract accepted for poster presentation at **Malvern Panalytical's 3rd Annual Pharma Conference** 11–12 June 2024, London, United Kingdom.

Modulating Silk Nanoparticle Size in Semi-Batch Format

Roamcharern, N.; Seib, F. P.; Rattray, Z.

Background: Biomaterials and synthetic polymers have been used in biomedical applications due to their useful biological and physical properties, including biodegradability, biocompatibility, and payload stability. Silk fibroin (SF) has emerged as a potential candidate for drug delivery due to its favourable characteristics governed by its tunable secondary structure. Therefore, the function of additive U.A. in modulating silk conformation may offer potential scope for improving silk nanoparticle (SNP) manufacture at the laboratory and industrial scales.

Methods: This study manufactured a SNP in semi-batch format in the presence of an additive (A.U. proprietary information). Briefly, the SF solution was mixed with the A.U., then added dropwise into isopropanol at a volume ratio of 1:5 mL. The resulting SNPs were analysed with dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA). Analysis of zeta potential was conducted using electrophoretic light scattering.

Results: U.A. showed interesting size tuning ability in SNP formulations contributing to increased particle size. DLS measured particle size was consistently smaller than NTA measurements, reporting a size at 290 d.nm and 173 d.nm, respectively. Interestingly, U.A. showed less impact on the polydispersity index (PDI); however the zeta potential ranged from –48 mV.

Conclusions: U.A. inclusion during SNP formulation had a great impact on SNP manufacture in the semi-batch format, enabling control of nanoparticle size and altering measured zeta potential. These findings can inform the use of U.A. as a strategy for modulating SNP physicochemical properties during manufacturing, providing SNPs with optimal characteristics.

Appendix E

Abstract accepted for poster presentation at **15th APS PharmSci 2024** 4–6 September 2024, Huddersfield, United Kingdom.

Coordinating Silk Nanoparticle Properties

Roamcharern, N.; Seib, F. P.; Rattray, Z.

Recently, silk fibroin has emerged as a viable material for drug delivery applications, providing a broad spectrum of silk format and application use. The goal of this study was to use spiking during silk nanoparticle (SNP) manufacture, with the aim to improve SNP physiochemical properties. We found that spiking of the additive (U.A.) influenced particle size of resulting SNP with less effect on SNP PDI and zeta potential. The pre-incubation of U.A. for 1 h prior to nanoprecipitation did not significantly impact on analysed physicochemical properties, which might imply high reproducibility for this manufacturing system.

Appendix F

Abstract accepted for poster presentation at **14th APS PharmSci 2023** 5–7 September 2023, Reading, United Kingdom.

Improved Semi-batch Silk Fibroin Nanoparticle Manufacture

Roamcharern, N.; Matthew, S. A. L.; Rattray, Z.; Seib, F. P.

Recently, silk Nanomedicine has the potential to overcome adverse effects associated with cytotoxic chemotherapy by altering physico-chemical-mediated tissue distribution to a ‘design-lead’ profile. Protein-based anticancer drug delivery has shown particular promise because it improved efficiency, bioavailability and pharmacokinetics of the payload. Recently, silk fibroin (SF) has emerged as an interesting protein-based nanocarrier because of its biochemical properties, including low toxicity, biocompatibility, biodegradability and flexible processing options. However, manufacturing these systems still requires further development. Herein, the SF nanoparticle (SNP) were manufactured using semi-batch format and corresponding physicochemical properties were characterized.