Expert Opinion

Current approaches to stabilising and analysing proteins during microencapsulation in PLGA

Christopher F van der Walle†, Gaurav Sharma & MNV Ravi Kumar
University of Strathclyde, Strathclyde Institute of Pharmacy and Biomedical Sciences, 27 Taylor Street, Glasgow, G4 0NR, UK

Background: Encapsulation of biomacromolecules in polyester micro- and nano-particles is now a routine procedure in many laboratories for achieving controlled and targeted delivery strategies. *Objectives*: Proteins are notoriously difficult to encapsulate without some degree of unfolding and loss of bioactivity, and this is despite around two decades of research. A case by case analysis appears necessary when determining which mechanism, generally unfolding at the emulsion interface or acidification of the particle interior, is most detrimental. The transient nature of the emulsion systems makes *in situ*, real-time analysis of interfacial events difficult, necessitating interpretation from model interfacial systems or analysis post-fabrication. *Methods*: The review will focus on: i) the emulsification efficiency of proteins, cf. interfacial adsorption; ii) current excipients and techniques used to stabilise proteins, outlining work towards the oral delivery of insulin as a case study; iii) analytical techniques used to characterise encapsulated protein. *Results*: There appears to be a recent trend towards the stabilization of proteins via direct complexation with polymers but, in contrast, emulsion techniques are emerging which err away from the use of stabilisers and/or excipients. A number of spectroscopic and spectrometric methods have found new application to the study of protein integrity in microspheres.

Keywords: excipients, insulin, PLGA, protein conformation, protein emulsification, spectrometry, spectroscopy

Expert Opin. Drug Deliv. (2009) ***6****(2):177-186*

1. Introduction

Among the biomolecular controlled-release delivery systems studied, encapsulation into biodegradable polyester particles of the micro or nano scale has generated particular interest. This is in part due to their promise of oral delivery regimes for proteins and DNA [1], and versatility in formulation for novel controlled-release systems that may also be administered via pulmonary and injectable routes. The biodegradable polyesters primarily used include poly(ε-caprolactone), poly(D,L-lactic acid) and, especially, poly(D,L-lactic-co-glycolic acid) (PLGA) [1]. This is due to their well-established low immunogenic effects, offering a biocompatible vehicle (reviewed in [2]); though there are more recent concerns over local, transient inflammation if the vehicles are implanted [3]. The ability to change the physicochemical and mechanical properties of the vehicles via selection of polymer molecular weight, co-polymerisation and functionalisation is particularly attractive. For example, various poly(ortho esters) have been synthesised with the intention to control polymer hydrolysis, with respect to hydrolytic rate and toxicity/acidity of degradation products, for use in DNA vaccine delivery vehicles [4]. It should be noted that the encapsulation of plasmid DNA in PLGA microspheres has recently been reviewed [5]. In recent years, a detailed understanding

informa healthcare

10.1517/17425240802680169 © 2009 Informa UK Ltd ISSN 1742-5247

of the physical chemistry of the polymeric matrices has emerged with benefits for particle engineering (reviewed in [6]) and control of the drug burst release (reviewed in [7]).

There exist several protocols to manufacture polyester microspheres, encapsulating a wide variety of therapeutic biomolecules. At the laboratory scale, the protocol may be as simple as the emulsification of a concentrated aqueous solution of protein, or the freeze-dried solid, in solvent followed by secondary emulsion in aqueous continuous phase: water-in-oil-in-water (w/o/w) or solid-in-oil-in-water (s/o/w) double emulsion–solvent evaporation. However, this apparent simplicity is misleading since there remain almost intractable problems of protein unfolding and degradation. These issues were identified rapidly during the development of PLGA microspheres as controlled-delivery vehicles for proteins [8] and remain relevant with respect to fabrication, storage and release. The review here is concerned with discussion of conformational changes: the loss of tertiary and secondary protein structure. Loss of conformation of a protein may not only be detrimental to the bioactivity (and therapeutic potential), but also cause immunogenic effects related to exposure of non-native peptide epitopes [9]. Maintaining protein conformation during encapsulation will be central to the development of a common strategy for biomolecule encapsulation, ideally avoiding the current need to adopt trial-and-error approaches for each new therapeutic target.

2. Emulsification efficiency of proteins

The interfacial activity of proteins is well understood and characterised [10] and relevant to their unfolding-aggregation during the emulsification step of a typical encapsulation protocol [11], or through adsorption to the polymeric matrix during release with concomitant loss [12]. However, in the absence of added surfactant (e.g., Tween) or emulsifier (e.g., polyvinyl alcohol), it is often overlooked that proteins themselves have generally good emulsification efficiency – that is, the primary w/o emulsion may be stabilised only because of the proteinaceous film at the interface. This of course is associated with significant loss of protein through unfolding-aggregation.

Our understanding of protein emulsifying properties is largely based on analysis of emulsifiers found in milk and seed [13], and until recently was largely phenomenological. Conformational criteria have, however, been characterised in detail for classical emulsifying proteins during adsorption-unfolding at the oil/water interface [11]. These changes promote hydrophobic and electrostatic interactions [14], and disulphide-mediated polymerisation where possible [15], which are thought to stabilise the viscoelastic protein film around oil droplets. Strongly emulsifying proteins are often composed of α-helical and/or random coil structure in aqueous solution, and may lose or gain α-helical structure during interfacial adsorption [13]. The interfacial activity of the globular protein myoglobin has been shown to be due to alignment of amphipathic α-helices at the interface, correlating with the calculated hydrophobic moment (a measure of peptide amphipathicity) [16]. Beta-lactoglobulin provides an example of a β-sheet protein with emulsifying activity, comprising mainly β-sheet and random coil but also α-helical structure [17]. Thus, secondary structure clearly plays a role in protein emulsification efficiency.

Modular proteins such as fibronectin also strongly adsorb to the oil–water interface, displacing bovine serum albumin (BSA, itself a protein with strong emulsification efficiency) even when at lower concentrations [18]. Since the modular β-sandwich motif is common to the immunoglobulin superfamily, an understanding of their mechanism will be of importance to pharmaceutical formulation via polymer encapsulation [19]. The *de novo* design of emulsifying properties on the β-sandwich motif has been reported [20]. This involved site-directed mutagenesis to increase the hydrophobic moment of individual β-strands within each modular domain, independently of the individual domain conformational stability, and without generating hydrophobic surface patches on the natively folded protein because the emulsifying role of such regions is well understood [21]. Surprisingly, domain–domain mobility and domain conformation stability were important parameters in determining the proteins' emulsification efficiency [20]. However, the drawback of this work was that only semi-empirical data for emulsion stability (droplet coalescence under static conditions) and emulsifying performance (change in droplet size under constant shear, as relevant to double emulsion–solvent evaporation) were obtained. Measurement of oil/water interfacial tensions would have allowed quantification of the force that acts on the protein at the interface.

Very recently, this experiment was performed using the pendant drop method, modelling the w/o primary emulsion while quantifying the adsorption kinetics and rheological properties of the proteinaceous film [22]. In the absence of surfactant, the model protein lysozyme adsorbed rapidly to the interface only after an initial lag phase during which protein molecules do not span the oil/water phases – that is, the decrease in surface tension starts only at the point when a particular protein monolayer coverage (determined by the adsorption parameters of the protein and o/w interface) has been reached. Whether or not this lag phase exists during homogenisation is uncertain and cannot be modelled using the pendant drop method. The rapid fall in surface tension would be explained by protein unfolding at the interface, possibly accompanied by helix–sheet transition (though this was not measured), and was followed by a slow fall in surface tension. The latter is generally accounted for by further relaxation (molecular rearrangement and change in the 'footprint') of the protein at the interface [23]. Acquisition of similar data using pendant drop method for various scenarios of the primary emulsion in PLGA encapsulation is expected to be a

productive route to the molecular characterisation of this complex interfacial system (**Table 1**).

3. Emerging techniques for the stabilisation of proteins during microencapsulation

3.1 PEGylation

Covalent modification of proteins with polyethylene glycol (PEG) of varying MW, 'PEGylation', is not commonly reported with regard to stabilisation during encapsulation but nevertheless leans on extensive data and methodology for the PEGylation of liposomes and proteins to increase circulation time. It is important to distinguish the use of PEGylated proteins from the simple addition of free PEG to the emulsification system, for reasons that may lie not only with the use of PEG as an emulsifier but also with its use in protein lyophilisation [24]. The rationale in PEGylating peptides during microencapsulation has primarily been to alter the release profile, overcoming problems with burst release [25,26]. Nevertheless, PEGylation of interferon-α with methoxy-PEG, MW 2000 and 5000, was observed to maintain an improved aqueous solubility (~ 90%) over the non-PEGylated form (~ 30%) following 60-s homogenisation in the presence of dichloromethane [26].

Translation of these data for PEGylated peptides to maintenance of protein conformational during encapsulation is not clear, partly due to the low number of reports. For example, high molar ratios of PEG:chymotrypsinogen (> 8) appeared to promote aggregation, whereas low molar ratios had the effect of minimising protein instability during encapsulation [27]. More widespread use of the PEGylation technique may depend on improved control of the PEGylation reaction, generating or purifying from a mixture clearly defined mono-PEG or multi-PEG protein species. Case-by-case analysis of the effect of PEGylation on pharmacological efficacy is also required.

One clear advantage of protein PEGylation has been reported for ribonuclease A (RNase A) adsorption to PLGA surfaces (**Table 1**) [28]. In this work, mono- and di-PEG-RNase A were separated by size-exclusion chromatography, fluorescently labelled and their adsorption to spin-coated PLGA surfaces analysed using total internal reflection fluorescence (TIRF). PLGA surface coverage (molecules per unit area) proceeded in the order: non-conjugated RNase A > mono-PEG-RNase A > di-PEG-RNase A. PEG-RNase A adsorption was transport-limited, as for PEG adsorption, but not RNase A. Therefore, the adsorption mechanism proposed was for an initial PLGA surface interaction via the pendent PEG groups (rapid adsorption phase) followed by protein–PLGA interaction. Since RNase A adsorption to PLGA was not transport-limited but controlled by hydrophobic (but not electrostatic) interactions, models of protein adsorption to silica (the more commonly used surface in TIRF) should not necessarily be applied to protein adsorption to PLGA microspheres [29]. Mono-PEG-RNase A adsorption to aged PLGA films was around half that for fresh PLGA surfaces, and this is interesting since the physical ageing of PLGA microspheres through β-relaxation of the polyester chains is known to occur for temperatures > 10°C [30].

3.2 Optimisation of the emulsion system and protein particles

From the discussion above, it is clear that one of the most detrimental steps to protein encapsulation is the primary w/o emulsion. It was quickly realised that non-aqueous emulsions systems could equally produce protein-loaded microspheres, with the work of Griebenow and colleagues directed to solid-in-oil-in-water (s/o/w) emulsification (reviewed in [31]). While the mobility of a dehydrated protein is much reduced, there are of course cryogenic and lyophilisation stresses on the protein beforehand and problems with higher residual solvent. There have, however, been some recent reports addressing these issues. An anhydrous solid-in-oil-in-oil (s/o/o) protocol has been reported [32] wherein excipient-free protein powders produced by spray-freezing were sonicated into acetonitrile, mixed with PLGA dissolved in acetonitrile, and stirred into paraffin oil. The percentage helix of the encapsulated albumin was around 34%, compared with 36% for a s/o/w protocol and 51% for the starting material. Although the protocol requires a relatively complex series of steps to initially reduce and then extract the acetonitrile, the excipient-free spray-freezing process is attractive in combination with microencapsulation and should be applicable to many proteins including immunoglobulins (**Table 1**). A standard spray-drying protocol was recently employed for w/o/w double emulsions, compared to more typical freeze-drying of microspheres, and shown to maintain structural integrity of the model protein [33]. It may be interesting to combine this approach with the spray-freezing process above for a wider range of proteins and peptides.

Given the high residual solvent content associated with s/o/w or s/o/o microencapsulation, one study investigated an optimum o:w ratio and alternative solvents to the commonly used dichloromethane and ethyl acetate [34]. While protein stability was improved by decreasing the time for microsphere hardening (solvent extraction into the continuous phase and evaporation), this did change microsphere morphology, probably because microsphere diameter is partially dependent on the secondary emulsion lifetime and stability during homogenisation [6]. Thus, changing the o:w ratio may not always be acceptable if specific microsphere diameters (e.g., for inhalation) are required. However, the less toxic butyl acetate was found to be a useful alternative to dichloromethane in PLGA microsphere fabrication. A recent, interesting approach to achieving the native protein conformation following microencapsulation is to encourage refolding of the precipitated protein dispersed in dichloromethane containing dissolved PLGA (0.6% w/w protein:PLGA ratio) [35]. Key to the success of the study appears to have been the

Table 1. Summary of protein instability issues and possible solutions.

Source of protein instability	Possible solutions	Example
Adsorption to the water/oil interface	Screening for alternative surfactants and optimization of surfactant concentration	[19]
	Use of s/o/w or s/o/o emulsion systems	[31, 32]
	Co-encapsulation with a protein that preferentially adsorbs to the interface	[18]
Protein aggregation and adsorption to the polymer matrix	Complexation with charged diblock co-polymers	[48, 49]
	Refolding of precipitated proteins	[35]
	PEGylation of proteins	[28]
Inherent emulsification efficiency	Site-directed mutagenesis of protein amphiphilic motifs	[20]
	Use of alternative solvents	[34]
Acidification of the polymer matrix	Incorporation of antacid	[44]
	Incorporation of co-polymers harbouring poly(L-histidine) block(s)	[48]

careful design of the conditions used (glycofurol with sodium chloride) for protein precipitation, which would otherwise be uncontrolled and lead to irreversible denaturation. However, issues of protein loss, burst release and possible denaturation of the protein during release remained but, as for the spray-freezing technique above, the process makes important steps to move away from stabilisers and/or excipients. Since there are many methods to precipitate protein in a controlled manner, including validated methods using supercritical carbon dioxide [36], the general principles of the technique may be explored further.

4. Current excipients used for protein microencapsulation

4.1 Surfactants and antacids

Despite the apparent trend for emerging techniques to avoid the use of excipients, for many research groups, the inclusion of stabilisers of the emulsion and excipients remains a necessity. Polyvinyl alcohol (PVA) was historically used to stabilise proteins during emulsification and this remains the case today, particularly for stabilisation of the secondary emulsion [37]. Alternative stabilisers such as Tweens® (ICI Americas Inc., USA) and Pluronics® (BASF Corp.,Germany) have been investigated [19] and their use continues [38], albeit on a rather ad-hoc basis. One report has attempted to relate surfactant hydrophile–lipophile balance (HLB) with stabilisation of the dichloromethane/water interface (w/o primary emulsion) [39]. Surprisingly, the Pluronic triblock co-polymers were universally poor stabilisers and sorbitan surfactants with a more hydrophilic nature (e.g., Tween 20) best stabilised the concave interfacial w/o curvature, implying solvation of the PEG head-group within the dichloromethane 'oil' phase. This is in contrast with their behaviour predicted by their high HLB values (> 10), which imply a preferential stabilisation of (non-volatile) oil-in-water emulsions, where the greater degree of solvation in the water phase leads to convex interfacial curvatures [40]. However, PEG is unusual in its ability to partition into both the dichloromethane and water phases; this is thought to be due to the PEG chain adjusting its conformation accordingly so as to more strongly interact with either apolar or polar solvents through selective intermolecular forces [41]. PEGs of varying MW have been investigated as stabilisers of the primary emulsion for protein encapsulation [42].

The acidic microenvironment of the polyester matrix encapsulating protein is well characterised as detrimental to protein stability, with the incorporation of antacids such as $Mg(OH)_2$ shown to maintain pH [43]. However, it is interesting that the use of this technique has not become more widespread, despite its apparent simplicity (**Table 1**). This may be because proteins simply don't reside in the microsphere interior for long enough to make the acidic breakdown products of polyesters a problem; this being a consequence of the burst release, reviewed elsewhere [7]. Possibly, only microspheres engineered to retain a major proportion of their protein load for up to several weeks would be expected to include an antacid excipient. This is the case for single-dose vaccines for antigen delivery from PLGA microspheres over 1 – 3 months, and here good examples of the need for $Mg(OH)_2$ to stabilise hepatitis B surface antigen [44] and $MgCO_3$ to stabilise tetanus toxoid can be found [45]. The latter study in particular made thorough analysis of tetanus toxoid conformational changes using circular dichroism for titration of $MgCO_3$ against pH. Both studies also used a variety of other protein stabilisers (e.g., trehalose, amino acids) during microencapsulation that have been thoroughly reviewed elsewhere [46].

4.2 Complex co-polymers

The group of Bae have synthesised novel PEG-poly(L-histidine) diblock co-polymers, which are intended to provide a 'temporal and reversible molecular shield' to retain protein stability and alter release kinetics [47]. The molecular complexation between the protein and PEG-poly(L-histidine) involves ionic bonding which is dependent on the charge of the poly(L-histidine) block, considering the pKa of the imidazole

group (6.5 – 7.0), the charge on the protein, which is dependent on its pI, and the buffer or environmental pH. The same considerations are also true when considering dissociation of the complex at physiological pH. Interestingly, the mechanism of stabilisation of complexed protein was initially suggested to be a consequence of buffering against the acidic pH of the PLGA matrix, as demonstrated by fluorescence microscopy using pH-sensitive fluorophores [47]. However, later work for the complexation of insulin also showed that PEG-poly(L-histidine) decreased aggregation of insulin at the dichloromethane/water interface (**Table 1**) [48]. Presumably then, protein stabilisation by PEG-poly(L-histidine) is through the interfacial activity of the PEG group, the antacid capacity of the poly(L-histidine) group and the formation of the complex itself. Since both insulin and PEG-poly(L-histidine) are positively charged below pH 5.5 and therefore repel (rather than interact, as would be the case for an acidic protein such as BSA), measurement of the zeta-potential was important in order to determine complexation via ionic interaction. Zeta-potential measurements close to zero were recorded at pH 5.5 for all weight ratios of insulin:PEG-poly(L-histidine), though pH 6.0 was selected for reasons of maximum insulin solubility and only at weight ratios of ≥ 1:2 was insulin aggregation significantly attenuated.

The same group have also synthesised a related PEG-polycation diblock co-polymer, poly(ethylene glycol)-block-oligo(vinyl sulfadimethoxine) and a PEG-polyanion diblock co-polymer, poly(ethylene glycol)-block-poly(l-aspartic acid) [49]. These co-polymers may offer complexation of a greater range of proteins, although their widespread acceptability are likely to depend on their advantages over PEG/antacid combinations versus the disadvantage of proving the safety of new molecular entities. In this respect, it is interesting that another group have employed chondroitin sulphate, a naturally occurring *O*-sulphonated glycosaminoglycan with repeating disaccharide units of glucuronic acid and *N*-acetylglucosamine, to bring about protein complexation via ionic interaction and stabilisation [50]. Similar to PEG-poly(L-histidine), measurement of the zeta-potential was important in determining complexation and increasing weight ratios of protein:chondroitin sulphate (to 1:4) decreased protein aggregation. Interestingly, subcutaneous implantation of PLGA microspheres co-encapsulating protein and chondroitin sulphate attracted fewer neutrophils to the implantation site than blank PLGA microspheres (also compare with [3]). Other glycosaminoglycans such as hyaluronic acid could be investigated and further *in vivo* studies comparing local foreign body responses to excipient-containing and excipient-free PLGA microspheres encapsulating protein would seem necessary.

5. Emerging methods for analysing microencapsulated proteins

There are of course many well-characterised analytical methods for studying protein conformation and molecular changes, including Fourier transform infrared spectroscopy (FTIR), circular dichroism (CD) and mass spectrometry (**Table 2**). Given that protein encapsulation in polyester microspheres is established, with vaccine formulations now entering clinical trials [51], it is becoming necessary to fully assess protein denaturation in a robust manner. It should be emphasised that the conformation of peptides is generally less important and therefore less of a concern during encapsulation. Simple analysis of the extent of protein fragmentation by gel electrophoresis alone, for example, is therefore inadequate and one would expect quantitative data generated by size-exclusion high-pressure liquid chromatography (SEC-HPLC), in combination with determination of the conformation [52]. Ideally, characterisation of protein conformation should be made in the solid state following microsphere drying (e.g., using FTIR or Raman spectroscopy, as reviewed in [53]) and in solution following release (e.g., using CD, as reviewed in [54]).

5.1 Calorimetry

With respect to polyester microspheres, differential scanning calorimetry (DSC) is most commonly used to assess the glass transition of the polymer, to determine possible plasticisation through the use of excipients, physical ageing [30], or gamma radiation effects [55]. However, DSC can also be used to assess protein stability in solution during repeated thermal scans, crudely, by measurement of the midpoint temperature of denaturation (T_m), area and shape analysis of the heat adsorption peak (reviewed in [56]). Usefully, for the target protein in solution, DSC can screen reasonably rapidly the effects of various excipients and concentrations on protein conformational stability [24]. The technique has immediate application to the study of protein stability either before or after emulsification in a w/o/w protocol, or following rehydration of the fabricated microspheres (**Table 2**). For example, following repeated thermal scanning of lysozyme in phosphate buffer, Kang and colleagues showed that lysozyme recovered from the aqueous phase after emulsification with dichloromethane retained its native conformation [57]. Interestingly, the addition of trehalose was not observed to increase lysozyme recovery following emulsification, which the authors suggested may have been due to lack to affinity at the oil/water interface. DSC analysis of lysozyme recovered following the same emulsification, but around 7% w/v PLGA in the oil phase showed a decrease in T_m from 77.4 to approximately 75.9°C (no cyclodextran) and 76.3°C (with cyclodextran) and would suggest some conformational change. The importance of shape analysis and fitting of the thermogram to various unfolding models was aptly demonstrated for DSC analysis of conformational changes to BSA during emulsification, since BSA is known to have two thermally independent domains [58]. Further, the appearance of a shoulder in the thermogram for thermal scans of BSA recovered from dichloromethane-water emulsions provided evidence of water-soluble aggregates that may otherwise have

Table 2. Summary of established and emerging techniques for the analysis of encapsulated proteins.

Technique	Application	Example
Established		
FTIR	Conformation of protein encapsulated in freeze-dried microspheres	[53]
CD	Conformation of protein released from microsphere	[54]
PAGE, HLPC and MS	Integrity of protein primary structure	[52]
Emerging		
'Solid state' CD	Conformational change upon protein surface adsorption	[65]
XPS and ToF-SIMS	Detection of low amounts of adsorbed protein	[62]
DSC	Scoping of protein stability in solution prior to emulsification	[57]

been missed by simple assessment of the insoluble protein fraction at the interface and spectroscopic analysis of the water-soluble fraction.

5.2 Surface analytical techniques using mass spectrometry and 'solid-state' circular dichroism

Since proteins have the propensity to adsorb to surfaces, it will be important to develop techniques able to study the surface-adsorbed conformation of proteins following microencapsulation. This is distinct from analysis of the bulk protein entrapped with the PLGA matrix but is important, for example, in the development of vaccines using particulate formulations of antigens [59]. More generally, surface analysis is also necessary in order to probe molecular- and meso-scale interfaces between the particle–particle, particle–cell and particle–tissue [60].

For the detection of low amounts of adsorbed protein, X-ray photoelectron spectroscopy (XPS) and time of flight secondary ion mass spectrometry (ToF-SIMS) have been shown to identify surface proteins at surface coverages between 0.1 ng/cm^2 (particularly ToF-SIMS) and 15 ng/cm^2, depending on the type of surface (**Table 2**) [61]. The techniques are established in the surface characterisation of solid particulates, providing identification of molecular species and, in the case of XPS, atomic concentration (quantitative analysis). Their application to the analysis of proteins adsorbed to PLGA microspheres or nanoparticles is likely to become more widespread on account of their sensitivity and also because they are specific to the outer molecular layer of a particle of around 5 – 10 nm (the 3 + 4 β-sandwich domain common to the IgG superfamily has dimensions of around 3 × 4 nm, for example). Chesko and co-workers applied XPS and ToF-SIMS to the identification of antigen adsorbed to the surface of PLGA microspheres, through detection of nitrogen species against the polyester background [62]. The identification and quantification of other molecular species including surfactants, counter ions and excipients was also possible. This would make XPS and ToF-SIMS useful not only for characterisation of protein adsorbed to the microsphere surface following a particular period of release, but also of residual surfactant following washing of the hardened microspheres [63].

Complementary to the identification and quantification of protein and other molecular species adsorbed to the particle surface, is the determination of the protein surface-adsorbed conformation. Spectroscopic studies of surface-adsorbed proteins are generally described for near optically clear nanoparticles with minimal light-scattering [64]. However, PLGA microspheres are generally > 1 μm and therefore to measure the secondary structure of adsorbed proteins by CD requires careful analysis: the validated methodology described by Ganesan and colleagues [65] involves an empirical approach wherein a correction factor is applied to the CD spectra in order to address the spectral distortion arising as a consequence of absorption flattening, due to the close-packing of immobilised chromophores at a surface [66]. Specific application of this technique to the study of PLGA microspheres has yet to be investigated. However, it is likely to prove useful since the data acquired provide semiquantitative approximation of the secondary structure composition, and can be used to probe protein unfolding as a function of solution conformational stability and surface hydrophobicity (**Table 2**) [29].

6. Case study: oral delivery of insulin

Insulin is a 51 amino acid peptide produced in pancreatic islets and is the principal modulator of glucose homeostasis in the body, the deficiency of which results in diabetes mellitus [67]. Despite the many developments in protein and peptide delivery via polymer based particulate systems, an efficient controlled delivery system satisfying the biological and chemical stability of insulin, an optimum bioavailability and patient compliance, still remains an elusive task. Since the pioneering work by Damgé and co-workers in 1988 [68], who reported a sustained hypoglycaemic action lasting up to 3 days in diabetic rats administered orally with insulin nanocapsules, a number of researchers have developed nanoparticle-based oral delivery systems for insulin. The beauty of these systems lies in their biocompatible and biodegradable nature, along with their ability to prolong drug release. The nature of the polymer used in fabricating these systems influences both the size and the characteristics of insulin release from these nanoparticles [69].

PLGA has been the most investigated biodegradable polymer, in part due to its approval in drug formulations and medical devices by the FDA and partly due to its versatility in terms of availability of different molecular weights, compositions and variable degradation rates for drug depots. PLGA-based

insulin nanoparticles (diameter < 200 nm) have been widely investigated for oral administration of insulin in diabetic animals where these delivery systems showed a marked decrease in plasma glucose levels post administration at different doses.

Carino and colleagues [70] reported insulin-based PLGA nanoparticles with fumaric anhydride oligomer and iron oxide additives (FAO/PLGA) for oral administration. The nanoparticles elicited reduced glycemia in response to a subcutaneous glucose tolerance test along with 11.4% efficacy in comparison to intraperitoneally administered insulin. Other researchers have developed chitosan-coated insulin nanoparticles that further increased the oral bioavailability of insulin by 15.4% compared with non-coated nanoparticles [71].

Complexing insulin with phosphatidylcholine, with the aim of enhancing its liposolubility in the oil phase via the generation of reverse micelles prior to solvent evaporation, has been investigated as a strategy to increase its entrapment in PLGA nanoparticles and improve its relative bioavailability by 7.7% [72]. In another protocol, the pH-sensitive cellulose copolymer HPMCP-55 was utilised to prevent the release of insulin in the stomach from PLGA nanoparticles; this increased the relative bioavailability of insulin by 6.27% [73].

During the nanoencapsulation process, insulin faces the same stress factors as broadly described above: denaturation followed by non-covalent aggregation, PLGA acidification leading to oligomerisation and aggregation; and also specific stress factors on account of its primary structure: soluble covalent dimer formation through disulfide shuffling and deamidation [74-76]. In addition, with the intention to develop a marketable medicine, the pharmaceutical scientist must also address formulation challenges that meet the criteria during long-term stability testing – which includes assessment of the mobility of both the polymer molecules and insulin molecules. UV spectrophotometry, size exclusion chromatography and reverse phase HPLC are routinely used techniques for assessment of insulin stability and entrapment in these systems.

Although HPLC methods predominate for quantitative assessment of insulin entrapment efficiency, release and oligomerisation, a recent study has developed a similarly quantitative method using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry using calibration against an Arg-insulin internal standard [77]. The immediate advantage of the mass spectrometry technique is the large reduction in material needed for analysis and that harsh extraction procedures involving alkali or solvents can be avoided, thereby not perturbing the structure or intermolecular interactions.

7. Regulatory considerations

The regulatory agencies are struggling hard to enforce strict (FDA) guidelines for nanotechnology products used in drug delivery that are based on a size range of 1 – 100 nm. To our understanding, such guidelines may be of limited use, since most of the polymeric and/or lipid drug delivery formulations consist of particles with sizes well over 100 nm. It is of utmost importance that the safety of nanoparticulate drug delivery vehicles be established, since the very small size of these particles is expected to facilitate their internalisation following ingestion. However, these issues have to be dealt with on an individual basis, rather than generalising across the various formulation platforms as seen today.

Safety is a relative term and is generally defined in terms of an upper maximum limit up to which a substance can be used (FDA's GRAS List, 'Generally Recognized As Safe'). This may be based on the toxicological data of use in other drug delivery preparations given by the same (oral) route. One particular challenge is that most of the excipients, solvents and polymeric matrix materials used in nanoparticle fabrication are not used in conventional medicines and therefore may not have precedent toxicology profiles. In addressing this challenge, the tissue distribution and cellular interaction of the nanoparticles needs to be studied on a short- as well as long term basis. It may further be necessary for these studies to be performed for each particular drug formulation developed because the toxicity of the medicine may also be exerted through the encapsulated drug in nanoparticulate form.

Drawing up guidelines for the regulation of protein nanoparticulate formulations is likely to be more complex than for small organic molecules, given the discussion in this review of protein conformational stability. Poor protein stability has the potential to exacerbate immunological responses, which may include toxicity but also the rate of clearance of the protein [9]. Thus, toxicological evaluation should be conducted for freshly prepared nanoparticulate formulations (with and without active ingredients), as for formulations subjected to stability studies.

All this said, it is encouraging that issues of potential toxicology of nanoparticulates are being considered in depth on the basis of experimental data (recently reviewed in [78]).

8. Expert opinion

The origin of protein instability during microencapsulation has proven difficult to define. Protein unfolding at the interface may have been partially solved by the use of s/o/w protocols, but only to be replaced by related problems of protein instability during rehydration and release. Imaginative approaches to overcoming the problems of efficient encapsulation without structural loss have been described; it has also been shown that it is possible to produce protein-loaded microspheres without the need for excipients at all stages of the process. This will be interesting from a regulatory viewpoint, since there are relatively few emulsifiers that have approval for human medicines. Conversely, ever more complicated stabilisers with multiple functions are being developed, with some success with regard to known major

mechanisms of protein instability – the acidic environment of the PLGA matrix and the oil/water interface during emulsification. The regulatory framework surrounding the assessment of particulate delivery vehicles is at an early stage and further progress is likely to require short- and long-term toxicological studies, particularly for nanoparticulates.

It is also being recognised that protein stresses may arise from the drying process, whether via lyophilisation or spray-drying. As a result, it is becoming increasingly popular to include lyo- and cryo-protectants that are well characterised, such as trehalose and PEGs. However, a clear trend regarding the absolute need for these additives has not yet emerged, except perhaps in the case of microsphere formulations of antigens as vaccines. Unfortunately, it appears that a case-by-case basis will be required, selecting the route of fabrication through consideration not only of microsphere size and morphology, but also of shelf-life and dosage regime. What is surprising is that there is relatively little work describing protein encapsulation through PLGA dissolved in supercritical carbon dioxide (sc CO_2). Much promise was originally associated with this technique on the basis of its omission of potentially toxic solvents. However, the use of hybrid sc CO_2-solvent protocols, along with the need for relatively complex and expensive equipment for handling sc CO_2, appears to have comprised this apparent potential.

It is noteworthy that, while there is a wealth of information regarding the molecular mechanisms of protein instability during lyophilisation, surface-adsorption and concentration, for example, there is little corresponding information for microencapsulation. However, in recent years, there have been noticeable advances in the understanding of the evolution of the protein- and/or surfactant-stabilised film at the solvent/water interface. Emerging spectroscopic and mass spectrometric techniques for the quantitative analysis of surface-adsorbed proteins may also be coupled to experimental protocols mimicking the transient nature of the secondary emulsion, in order to understand the dynamic process of microsphere precipitation and hardening. As long as there remain important therapeutic goals, such as the oral delivery of proteins, it is likely that this will remain a stimulating and productive field.

Declaration of interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers

1. Mathiowitz E, Jacob JS, Jong YS, et al. Biologically erodable microspheres as potential oral drug delivery systems. Nature 1997;386:410-4
2. Shive MS, Anderson JM. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliv Rev 1997;28:5-24
3. Kang SW, Cho ER, Jeon O, et al. The effect of microsphere degradation rate on the efficacy of polymeric microspheres as bulking agents: an 18-month follow-up study. J Biomed Mater Res B Appl Biomater 2007;80:253-9
4. Wang C, Ge Q, Ting D, et al. Molecularly engineered poly(ortho ester) microspheres for enhanced delivery of DNA vaccines. Nat Mater 2004;3:190-6
5. Abbas AO, Donovan MD, Salem AK. Formulating poly(lactide-co-glycolide) particles for plasmid DNA delivery. J Pharm Sci 2008;97:2448-61
6. Mohamed F, van der Walle CF. Engineering biodegradable polyester particles with specific drug targeting and drug release properties. J Pharm Sci 2008;97:71-87
7. Allison SD. Analysis of initial burst in PLGA microparticles. Exp Opin Drug Del 2008;5:615-28
8. van de WeertM, Hennink WE, Jiskoot W. Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm Res 2000;17:1159-67
9. Hermeling S, Crommelin DJ, Schellekens H, et al. Structure-immunogenicity relationships of therapeutic proteins. Pharm Res 2004;21:897-903
10. Norde W. Adsorption of proteins from solution at the solid-liquid interface. Adv Colloid Interface Sci 1986;25:267-340
11. van de Weert M, Hoechstetter J, Hennink WE, et al. The effect of a water/organic solvent interface on the structural stability of lysozyme. J Control Release 2000;68:351-9
12. Calis S, Jeyanthi R, Tsai T, et al. Adsorption of salmon-calcitonin to PLGA microspheres. Pharm Res 1995;12:1072-6
13. Burnett GR, Rigby NM, Mills ENC, et al. Characterization of the emulsification properties of 2S albumins from sunflower seed. J Colloid Interf Sci 2002;247:177-85
14. Damodaran S. Protein stabilization of emulsions and foams. J Food Sci 2005;70:R54-66
15. Dickinson E, Matsumura Y. Time-dependent polymerization of beta-lactoglobulin through disulfide bonds at the oil-water interface in emulsions. Int J Biol Macromols 1991;13:26-30
16. Poon S, Clarke A, Currie G, et al. Influence of alpha-helices on the emulsifying properties of proteins. Biosci Biotechnol Biochem 2001;65:1713-23
17. Qin BY, Bewley MC, Creamer LK, et al. Structural basis of the tanford transition of bovine beta-lactoglobulin. Biochemistry 1998;37:14014-23
18. Vaidya SS, Ofoli RY. Adsorption and interaction of fibronectin and human serum albumin at the liquid-liquid interface. Langmuir 2005;21:5852-8
19. Bouissou C, Potter U, Altroff H, et al. Controlled release of the fibronectin central cell binding domain from polymeric microspheres. J Control Release 2004;95:557-66
20. Annan WS, Fairhead M, Pereira P, et al. Emulsifying performance of modular beta-sandwich proteins: the hydrophobic moment and conformational stability. Protein Eng Des Sel 2006;19:537-45
• **De-novo design of emulsifying β-sandwich proteins.**
21. Toren A, Segal G, Ron EZ, et al. Structure-function studies of the recombinant protein bioemulsifier AlnA. Environ Microbiol 2002;4:257-61

22. Malzert-Freon A, Benoit JP, Boury F. Interactions between poly(ethylene glycol) and protein in dichloromethane/water emulsions: a study of interfacial properties. Eur J Pharm Biopharm 2008;69:835-43
•• **Rheological properties of the proteinaceous film for water-oil emulsions.**

23. Santore MM, Wertz CF. Protein spreading kinetics at liquid-solid interfaces via an adsorption probe method. Langmuir 2005;21:10172-8

24. Pereira P, Kelly SM, Cooper A, et al. Solution formulation and lyophilisation of a recombinant fibronectin fragment. Eur J Pharm Biopharm 2007;67:309-19

25. Park EJ, Na DH, Lee KC. In vitro release study of mono-PEGylated growth hormone-releasing peptide-6 from PLGA microspheres. Int J Pharm 2007;343:281-3

26. Diwan M, Park TG. Stabilization of recombinant interferon-alpha by pegylation for encapsulation in PLGA microspheres. Int J Pharm 2003;252:111-22

27. Castellanos IJ, Al-Azzam W, Griebenow K. Effect of the covalent modification with poly(ethylene glycol) on alpha-chymotrypsin stability upon encapsulation in poly(lactic-co-glycolic) microspheres. J Pharm Sci 2005;94:327-40
• **Encapsulation of PEGylated protein into PLGA microspheres.**

28. Daly SM, Przybycien TM, Tilton RD. Adsorption of poly(ethylene glycol)-modified ribonuclease A to a poly(lactide-co-glycolide) surface. Biotechnol Bioeng 2005;90:856-68
• **Decreased protein adsorption to PLGA surfaces via PEGylation.**

29. Pereira P, Kelly SM, Gellert PR, et al. Interdomain mobility and conformational stability of type III fibronectin domain pairs control surface adsorption, desorption and unfolding. Colloid Surfaces B 2008;64:1-9

30. RouseJJ, Mohamed F, van der Walle CF. Physical ageing and thermal analysis of PLGA microspheres encapsulating protein or DNA. Int J Pharm 2007;339:112-20

31. Perez C, Castellanos IJ, Costantino HR, et al. Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers. J Pharm Pharmacol 2002;54:301-13

32. Leach WT, Simpson DT, Val TN, et al. Uniform encapsulation of stable protein nanoparticles produced by spray freezing for the reduction of burst release. J Pharm Sci 2005;94:56-69
• **Excipient-free protein powders for s/o/o protocols.**

33. Lane ME, Brennan FS, Corrigan OI. Influence of post-emulsification drying processes on the microencapsulation of Human Serum Albumin. Int J Pharm 2006;307:16-22

34. Castellanos IJ, Griebenow K. Improved alpha-chymotrypsin stability upon encapsulation in PLGA microspheres by solvent replacement. Pharm Res 2003;20:1873-80
• **Assessment of alternative, less toxic surfactants for microencapsulation.**

35. Giteau A, Venier-Julienne MC, Marchal S, et al. Reversible protein precipitation to ensure stability during encapsulation within PLGA microspheres. Eur J Pharm Biopharm 2008;70:127-36
•• **Controlled protein precipitation and refolding.**

36. Okamoto H, Danjo K. Application of supercritical fluid to preparation of powders of high-molecular weight drugs for inhalation. Adv Drug Deliv Rev 2008;60:433-46

37. Lad MD, Birembaut F, Matthew JM, et al. The adsorbed conformation of globular proteins at the air/water interface. Phys Chem Chem Phys 2006;8:2179-86

38. Wei G, Lu LF, Lu WY. Stabilization of recombinant human growth hormone against emulsification-induced aggregation by Pluronic surfactants during microencapsulation. Int J Pharm 2007;338:125-32

39. Duncan G, Jess TJ, Mohamed F, et al. The influence of protein solubilisation, conformation and size on the burst release from poly(lactide-co-glycolide) microspheres. J Control Release 2005;110:34-48

40. Binks BP. Modern aspects of emulsion science. In: Binks B, editor, Modern aspects of emulsion science. Royal Society of Chemistry, 1998. p. 176-85

41. Harris JM. Introduction to biotechnical and biomedical applications of poly(ethylene glycol). In: Harris JM, editor. Poly(ethylene glycol) chemistry. New York: Plenum Press; 1992. p. 1-14

42. Castellanos IJ, Flores G, Griebenow K. Effect of the molecular weight of poly(ethylene glycol) used as emulsifier on alpha-chymotrypsin stability upon encapsulation in PLGA microspheres. J Pharm Pharmacol 2005;57:1261-9

43. Zhu G, Mallery SR, Schwendeman SP. Stabilization of proteins encapsulated in injectable poly (lactide- co-glycolide). Nat Biotechnol 2000;18:52-7

44. Jaganathan KS, Singh P, Prabakaran D, et al. Development of a single-dose stabilized poly(D,L-lactic-co-glycolic acid) microspheres-based vaccine against hepatitis B. J Pharm Pharmacol 2004;56:1243-50

45. Jiang W, Schwendeman SP. Stabilization of tetanus toxoid encapsulated in PLGA microspheres. Mol Pharmaceutics 2008;5:808-17

46. Bilati U, Allemann E, Doelker E. Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles. Eur J harm Biopharm 2005;59:375-88

47. Kim JH, Taluja A, Knutson K, et al. Stability of bovine serum albumin complexed with PEG-poly(L-histidine) diblock copolymer in PLGA microspheres. J Control Release 2005;109:86-100
•• **Charged co-polymers as 'temporal and reversible molecular shields'.**

48. Taluja A, Bae YH. Role of a novel excipient poly(ethylene glycol)-b-poly(L-histidine) in retention of physical stability of insulin at aqueous/organic interface. Mol Pharmaceutics 2007;4:561-70

49. Taluja A, Bae YH. Role of a novel multifunctional excipient poly(ethylene glycol)-block-oligo(vinyl sulfadimethoxine) in controlled release of lysozyme from PLGA microspheres. Int J Pharm 2008;358:50-9

50. Lee ES, Park KH, Kang D, et al. Protein complexed with chondroitin sulfate in poly(lactide-co-glycolide) microspheres. Biomaterials 2007;28:2754-62

51. Katz DE, Delorimier AJ, Wolf MK, et al. Oral immunization of adult volunteers with microencapsulated enterotoxigenic Escherichia coli (ETEC) CS6 antigen. Vaccine 2003;21:341-6

52. Wang J, Chua KM, Wang CH. Stabilization and encapsulation of human immunoglobulin G into biodegradable microspheres. J Colloid Interface Sci 2004;271:92-101

53. Tantipolphan R, Rades T, Medlicott NJ. Insights into the structure of protein by

vibrational spectroscopy. Current Pharm Anal 2008;4:53-68

54. Kelly SM, Jess TJ, Price NC. How to study proteins by circular dichroism. Biochim Biophys Acta 2005;1751:119-39

55. Carrascosa C, Espejo L, Torrado S, et al. Effect of gamma-sterilization process on PLGA microspheres loaded with insulin-like growth factor-I (IGF-I). J Biomater Appl 2003;18:95-108

56. Cooper A, Thermodynamics of protein folding and stability. In: Allen G, editor. Protein: a comprehensive treatise, vol. 2. Starnford, CT: JAI Press Inc; 1999. p. 217-70

57. Kang F, Jiang G, Hinderliter A, et al. Lysozyme stability in primary emulsion for PLGA microsphere preparation: effect of recovery methods and stabilizing excipients. Pharm Res 2002;19:629-33

• **T_m characterisation of protein by DSC to inform the microencapsulation protocol.**

58. Kang F, Singh J. Conformational stability of a model protein (bovine serum albumin) during primary emulsification process of PLGA microspheres synthesis. Int J Pharm 2003;260:149-56

59. Yang YW, Hsu PY. The effect of poly(D,L-lactide-co-glycolide) microparticles with polyelectrolyte self-assembled multilayer surfaces on the cross-presentation of exogenous antigens. Biomaterials 2008;29:2516-26

60. Peetla C, Labhasetwar V. Biophysical characterization of nanoparticle-endothelial model cell membrane interactions. Mol Pharmaceutics 2008;5:418-29

61. Wagner MS, Mcarthur SL, Shen M, et al. Limits of detection for time of flight secondary ion mass spectrometry (ToF-SIMS) and X-ray photoelectron spectroscopy (XPS): detection of low amounts of adsorbed protein. J Biomater Sci Polym Ed 2002;13:407-28

62. Chesko J, Kazzaz J, Ugozzoli M, et al. Characterization of antigens adsorbed to anionic PLG microparticles by XPS and TOF-SIMS. J Pharm Sci 2008;97:1443-53

•• **Detection of low amounts of protein adsorbed to microparticles.**

63. Sahoo SK, Panyam J, Prabha S, et al. Residual polyvinyl alcohol associated with poly (D,L-lactide- co-glycolide) nanoparticles affects their physical properties and cellular uptake. J Control Rel 2002;82:105-14

64. Koutsopoulos S, Tjeerdsma AM, Lieshout JF, et al. In situ structure and activity studies of an enzyme adsorbed on spectroscopically undetectable particles. Biomacromolecules 2005;6:1176-84

65. Ganesan A, Price NC, Kelly SM, et al. Circular dichroism studies of subtilisin Carlsberg immobilised on micron sized silica particles. Biochim Biophys Acta 2006;1764:1119-25

• **Characterisation of surface-adsorbed protein conformation.**

66. Castiglioni E, Abbate S, Longhi G, et al. Wavelength shifts in solid-state circular dichroism spectra: a possible explanation. Chirality 2007;19:491-6

67. Banting FG, Best CH, Collip JB, et al. Pancreatic extracts in the treatment of diabetes mellitus. Preliminary report. Canadian Med Assoc J 1922;12:141-6

68. Damgé C, Michel C, Aprahamian M, et al. New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. Diabetes 1988;37:246-51

69. Damgé C, Reis CP, Maincent P. Nanoparticle strategies for the oral delivery of insulin. Expert Opin Drug Deliv 2008;5:45-68

70. Carino GP, Jacob JS, Mathiowitz E. Nanosphere based oral insulin delivery. J Control Release 2000;65:261-9

71. Pan Y, Li YJ, Gao P, et al. [Enhancement of gastrointestinal absorption of chitosan-coated insulin-loaded poly (lactic-co-glycolic acid) nanoparticles]. Yao Xue Xue Bao 2003;38:467-70

72. Cui F, Shi K, Zhang L, et al. Biodegradable nanoparticles loaded with insulin-phospholipid complex for oral delivery: preparation, in vitro characterization and in vivo evaluation. J Control Release 2006;114:242-50

73. Cui FD, Tao AJ, Cun DM, et al. Preparation of insulin loaded PLGA-Hp55 nanoparticles for oral delivery. J Pharm Sci 2007;96:421-7

74. Kwon YM, Baudys M, Knutson K, et al. In situ study of insulin aggregation induced by water-organic solvent interface. Pharm Res 2001;18:1754-9

75. Rosa GD, Iommelli R, La Rotonda MI, et al. Influence of the co-encapsulation of different non-ionic surfactants on the properties of PLGA insulin-loaded microspheres. J Control Release 2000;69:283-95

76. Shao PG, Bailey LC. Porcine insulin biodegradable polyester microspheres: stability and in vitro release characteristics. Pharm Dev Technol 2000;5:1-9

77. Bilati U, Pasquarello C, Corthals GL, et al. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for quantitation and molecular stability assessment of insulin entrapped within PLGA nanoparticles. J Pharm Sci 2005;94:688-94

78. Linkov I, Satterstrom FK, Corey LM. Nanotoxicology and nanomedicine: making hard decisions. Nanomedicine 2008;4:167-71

Affiliation

Christopher F van der Walle†, Gaurav Sharma & MNV Ravi Kumar
†Author for correspondence
University of Strathclyde,
Strathclyde Institute of Pharmacy and Biomedical Sciences,
27 Taylor Street, Glasgow, G4 0NR, UK
Tel: +44 141 548 5755; Fax: +44 141 552 2562;
E-mail: chris.walle@strath.ac.uk