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Rheopexy of synovial fluid and protein aggregation

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Bovine synovial fluid and albumin solutions of similar concentration are rheopectic (stress increases with time in steady shear). This unusual flow characteristic is caused by protein aggregation, and the total stress is enhanced by entanglement of this tenuous protein network with the long-chain polysaccharide sodium hyaluronate under physiological conditions. Neutron scattering measurements on albumin solutions demonstrate protein aggregation and all measurements are consistent with a weak dipolar attraction energy (of order $3kT$) that is most likely augmented by hydrophobic interactions and/or disulfide bond formation between proteins. Protein aggregation appears to play an important role in the mechanical properties of blood and synovial fluid. We also suggest a connection between the observed rheopexy and the remarkable lubrication properties of synovial fluid.

Keywords: hyaluronic acid; depletion flocculation; dipolar attraction; plasma proteins

1. INTRODUCTION

Synovial fluid lubricates motion of mammalian freely moving joints, as well as supplying tissues with nutrients and removing catabolic products (McCutchen 1978). Normal synovial joints exhibit an extremely low coefficient of friction, *similar to an ice skate on ice* (Martin *et al.* 1998), and their cartilage does not abrade over several decades (Mori *et al.* 2002). While this is due in part to the inherent ability of biological systems to self-maintain and self-repair, the effective lubrication of these joints also plays a significant role in their longevity. The coefficient of friction of bovine synovial fluid, when measured using excised canine joints, is $\mu = 0.002$–0.01 (Linn 1968; Mabuchi *et al.* 1994). When simple saline is used in these joints, the friction coefficient increases by 300% to $\mu = 0.01$–0.03 (Linn 1968; Mabuchi *et al.* 1994). Adding 10 mg ml^{-1} hyaluronic acid to the saline only lowers the friction coefficient a small amount ($\mu = 0.005$–0.02) strongly indicating that other components of synovial fluid play important roles in lubrication (Linn 1968; Mabuchi *et al.* 1994). For comparison, a typical automotive motor oil has a friction coefficient $\mu =$ 0.01–0.05 when measured between steel surfaces (Tarasov *et al.* 2002), similar to the saline solution with no polymer present.

Despite considerable research, the manner in which synovial fluid acts as such a good lubricant is not understood. Lubrication in moving joints may be controlled by the mechanical or surface properties of articular cartilage, or the macromolecules of synovial fluid, or some complex combination of these. Swann astutely suggests that lubrication of freely moving joints 'should be thought of as a continuous spectrum of tissue, cellular, and molecular events and interactions, which contribute in varying degrees at various times' (Swann 1978).

Synovial joints sustain load bearing motion for a lifetime and in healthy joints, minimal damage occurs to the cartilage that covers bone surfaces. This is not the case for arthritic joints. Nearly 70 million Americans, *one out of every three adults*, are affected by arthritis or chronic joint symptoms (Bolen *et al.* 2002). As the population ages, this number will continue to grow dramatically. Arthritis already is the leading cause of disability in the United States (Bolen *et al.* 2002). Arthritis comprises over 100 different diseases and conditions, of which the most common are osteoarthritis (OA) and rheumatoid arthritis (RA) (Bolen *et al.* 2002), which, according to the Arthritis Foundation web site (www.arthritis.org) affect roughly 21 million and 2.1 million Americans, respectively. In comparison to healthy synovial fluid, diseased fluid has a reduced viscosity (Johnston 1955; Bloch & Dintenfass 1963; Ferguson *et al.* 1968; Davies & Palfrey 1968; Anadere *et al.* 1979; Chmiel & Walitza 1980; Schurz & Ribitsch 1987; Safari *et al.* 1990; Gomez & Thurston

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1993; Schurz 1996). In OA, this reduction in viscosity is particularly severe, and results from a decline in both the molecular weight and concentration of hyaluronic acid (Mori *et al.* 2002).

The most abundant macromolecules in synovial fluid are the sodium salt of hyaluronic acid (NaHA, *ca* 3 mg ml^{-1}, a high molecular weight anionic polysaccharide) and blood plasma proteins (albumin, *ca* 11 mg ml^{-1} and globulins, *ca* 7 mg ml^{-1}). In early studies of synovial fluid, many researchers tried to separate these macromolecules, but obtaining protein-free samples of NaHA proved impossible. As a result, the plasma proteins were incorrectly assumed to bind irreversibly to NaHA, forming a 'NaHA–protein complex' (Ogston & Stanier 1950, 1952). Evidence contradicting this assertion has recently been presented by Dubin *et al.* (Grymonpré *et al.* 2001), who showed that bovine serum albumin (BSA) (the most abundant protein in bovine synovial fluid) binds to NaHA at low pH (Xu *et al.* 2000), but not above pH = 5. Albumin shows similar behavior with other anionic polyelectrolytes (Matsudo *et al.* 2003; Sotiropoulou *et al.* 2005), only binding at low pH, where albumin has a net positive charge. These results clearly suggest that at physiological conditions (pH = 7.4), binding between NaHA and BSA in synovial fluid is highly unlikely.

The synovium that separates synovial fluid from plasma allows small globular proteins (albumin and globulins) to freely interchange between blood and synovial fluid. Yet, rheumatoid arthritis patients simultaneously have a higher than normal albumin concentration in their synovial fluid (Chmiel & Walitza 1980; Simkin 1997) and a smaller than normal albumin concentration in their plasma (Pigman *et al.* 1961). This observation strongly suggests that albumin binds to something in synovial fluid; herein we suggest that albumin binds to itself, forming multi-protein polymeric aggregates.

Early rheological studies of synovial fluid focused on shear thinning, and attempts were made to understand how this related to lubrication. Many studies used shear rates from 100 up to 3000 s^{-1}, much higher than those experienced during joint motion, although some studies do use lower rates (Davies & Palfrey 1968; Fouissac *et al.* 1993; Mo & Nishinari 2001; Mazzucco *et al.* 2002). At such high shear rates, the rheology of NaHA solutions and synovial fluid are very similar, leading to the conclusion that the mechanical properties of synovial fluid were controlled exclusively by the very high molecular weight NaHA (Sundblad 1953; Balazs & Sundblad 1959; ScottBlair 1974; Chmiel & Walitza 1980). This motivated many studies of NaHA solution rheology by Balazs (Balazs & Laurent 1951; Balazs *et al.* 1967; Gibbs *et al.* 1968; Balazs 1970), Nishinari (Kobayashi *et al.* 1994; Mo *et al.* 1999; Mo & Nishinari 2001), Rinaudo (Fouissac *et al.* 1993; Berriaud *et al.* 1994; Milas *et al.* 2001; Gatej *et al.* 2005) and others (Johnson *et al.* 1971; Morris *et al.* 1980; Welsh *et al.* 1980; Bothner & Wik 1987; Yanaki & Yamaguchi 1990, 1994; De Smedt *et al.* 1993; Lapcik *et al.* 1998). However, Boger (Tirtaatmadja *et al.* 1984) observed that proteins contributed greatly to the mechanical properties of synovial fluid, and combining synthetic NaHA with proteins has been shown to significantly alter the rheological properties at low shear rates (Oates *et al.* 2002). Many of the early rheology studies on NaHA solutions used NaHA from natural sources (such as rooster combs) that is believed to be contaminated with small amounts of protein (Balazs & Sundblad 1959; Tirtaatmadja *et al.* 1984; Milas *et al.* 2001), and such solutions lead to the incorrect conclusion that NaHA forms reversible gels that are strongly viscoelastic (Johnson *et al.* 1971; Welsh *et al.* 1980; Yanaki & Yamaguchi 1990; De Smedt *et al.* 1993). High molar mass NaHA from bacterial sources appears to be protein-free and behaves like any flexible polyelectrolyte in aqueous salt solutions (Fouissac *et al.* 1993; Milas *et al.* 2001; Krause *et al.* 2001), with no hints of associations or gel formation.

Synovial fluid rheology has long been utilized in the study of rheumatic diseases (ScottBlair *et al.* 1954). The literature on this topic is vast and a review is beyond the scope of this paper. However, significantly lower viscosities are often noted for fluids of both OA and RA patients (Johnston 1955; Bloch & Dintenfass 1963; Davies & Palfrey 1968; Ferguson *et al.* 1968; Anadere *et al.* 1979; Chmiel & Walitza 1980; Schurz & Ribitsch 1987; Safari *et al.* 1990; Gomez & Thurston 1993; Schurz 1996). A common theme in the interpretation of that observation is a change in the 'associations' or 'macromolecular complexes' in synovial fluid (Myers *et al.* 1966; Ferguson *et al.* 1968; Nuki & Ferguson 1971; Anadere *et al.* 1979; Schurz & Ribitsch 1987). However, what is not clear is which macromolecules are involved in such complexes.

In this paper, both bovine synovial fluid and solutions of BSA in phosphate-buffered saline (PBS) are shown to be *rheopectic*: stress grows in time during steady shear. Aggregation of BSA was seen using small-angle neutron scattering (SANS) of BSA solutions in D_2O (with PBS). The aggregation occurs under quiescent conditions as well as under shear. The aggregation is enhanced by the NaHA polyelectrolyte present in synovial fluid, although equilibrium dialysis suggests the interaction between NaHA and BSA is strictly repulsive.

2. EXPERIMENTAL

Bovine synovial fluid was obtained from stifle joints shortly after sacrifice. The samples were frozen for storage prior to measurement, but no further alterations were made to the fluid. The freezing has been shown not to change the rheology of these fluids (Oates 2002) and fluids from the stifle joints of two animals show considerable variation in rheology (although all four are rheopectic).

The bacterially synthesized sodium salt of hyaluronic acid (NaHA) was obtained from Genzyme (Certain commercial materials and equipment are identified in this paper in order to specify adequately the experimental procedure) (4876-02) with protein content less than 0.1% and $M = 1.5 \times 10^6$ (Krause *et al.* 2001). A model synovial fluid was developed (Krause 2000), containing NaHA, BSA (Sigma Fraction V, A-3059), and bovine γ-globulins (Sigma Fraction II, III, G-5009)

Table 1. Composition of the fluids used in the study, and typical human synovial fluid.

	NaHA (mg ml^{-1})	protein (albumin/γ-globulins) (mg ml^{-1})	cells (cells μl^{-1})
typical human synovial fluid	3	18 (11/7)	<100
our synovial fluid model	3	18 (11/7)	0
bovine synovial fluid of figure 1	—[a]	19[a]	<100
bovine serum albumin of figures 1–3	0	11 (11/0)	0

[a] Only the total protein content and cell content were determined for the bovine synovial fluid.

in PBS (pH = 7.4, 0.138 M NaCl and 0.0027 M KCl from Sigma). The concentrations of macromolecules and cells in the model synovial fluid and the bovine synovial fluid, as well as typical concentrations of macromolecules in human synovial fluid (which vary considerably) are summarized in table 1. Other macromolecules present in synovial fluid are at concentrations less than 1 mg ml^{-1}. The model synovial fluid solutions and all protein solutions were prepared in PBS *without stirring*. This was done to prevent foaming that can occur on vigorous stirring while dissolving proteins in water.

All rheological measurements were made using a Contraves LS30 viscometer with a stainless steel concentric cylinder geometry (outer cup diameter 12.0 mm, inner bob diameter 11.1 mm), operating at steady shear rates determined by the rotation speed of the cup. The air/liquid interface is far above the bob, where the gap between the cup and the spindle supporting the bob is large. As a consequence, the air–water interface makes a negligible contribution to the measured torque (less than one part in 10^4). The total sample volume is 2.5 ml, and any drying that occurs during measurement should make negligible contributions to the measured torque (the air–water interface was probed with a pin to ensure that no polymer film ever forms). The torque on the bob is measured by application of a compensating torque via a drag cup motor located between the bob and a torsion wire. This device can measure the viscosity of air to three significant figures at 100 s^{-1}. Calibration was made with Newtonian standard oils (and water) which all showed stress rapidly build up to a steady state value at each shear rate (between 0.01 and 100 s^{-1}). All solutions were presheared at 100 s^{-1} for 5 min after loading into the concentric cylinder geometry. This is a standard procedure in rheological measurements on 'interesting' liquids, aiming to standardize all solution loadings by 'erasing' any shear history effects during sample preparation and loading. As we shall see below, this preshear certainly does *not* erase the shear history and in particular is not sufficient to obtain reproducible results with any solutions we have studied containing BSA. With most fluids, steady shear results in the stress building with time and levelling-off at a steady state value, from which the viscosity is calculated. Solutions that we studied containing BSA only show steady state viscosities at shear rates exceeding 10 s^{-1}. At lower shear rates, no steady state value of the stress (or viscosity) is attained in our experiments.

Equilibrium dialysis (Ogston & Phelps 1960; Tinico *et al.* 2002) was used to assess the extent of binding between NaHA and BSA in PBS. A 5 ml dispodialyzer (Spectrum Labs #135582) that passes species with $M < 300\,000$ was filled with a solution of NaHA ($c = 0.3$–6 mg ml^{-1}) and immersed in a 1 l solution of BSA (0.25–11 mg ml^{-1}) in PBS. The membrane easily passes BSA ($M = 66\,000$), and the BSA equilibrates in roughly ten days (Oates 2002) to have equilibrium concentrations of BSA inside (with NaHA) and outside (no NaHA) the dispodialyzer. The concentrations of protein inside and outside the dispodialyzer after ten days were determined using standard protein assays: small quantities of solution were mixed with 5 ml of a dye solution (Pierce, Coomassie protein assay reagent #23200) and absorbance measured at 595 nm wavelength. Protein concentration was determined from separate calibration curves at each NaHA concentration studied (Oates 2002).

SANS measurements on BSA solutions in D_2O (with PBS) were performed at the NIST Center for Neutron Research on the NG-7 30 m SANS beamline. Three configurations were employed to measure low, intermediate and high scattering wavevectors, $q = (4\pi/\lambda)\sin(\theta/2)$, where λ is the neutron wavelength and θ is the scattering angle. In the low q configuration, $\lambda = 8.44$ Å, a sample to detector distance, $L = 11.8$ m, and a series of focusing lenses (Choi 2000) defined a q range of 0.005–0.008 nm^{-1}. The intermediate and high q configurations used $\lambda = 10.0$ Å, $L = 15$ m and $\lambda = 6.0$ Å, $L = 3.8$ m, respectively. The intermediate and high q configurations used circular apertures to collimate the beam. In all configurations, the wavelength spread was set to $\Delta\lambda/\lambda = \pm 0.11$. The samples consisted of solutions placed between quartz windows with a 1.0 mm gap, resulting in a scattering volume of 1.2 mm^3. Scattered intensity was collected on a two-dimensional CCD detector, corrected for backgrounds, and radially averaged. Background subtraction was achieved using the scattered intensity from D_2O with PBS in the same cell. Intensity is placed on an absolute scale through comparisons of the direct beam flux, and normalized by the scattering volume.

3. RESULTS

Rheopexy is defined as an increasing stress as a function of time during shear at a constant rate. At low shear rates ($\dot{\gamma} \leq 10$ s^{-1}), the bovine synovial fluid, the model fluid, and a solution of 11 mg ml^{-1} BSA in PBS all exhibit rheopexy as shown in figure 1. Similar rheopexy has previously been observed in synovial fluid at temperatures below 20 °C (O'Neill & Stachowiak 1996) and in some protein solutions (Rha & Pradipasena 1986; Renard *et al.* 1996). While figure 1 shows

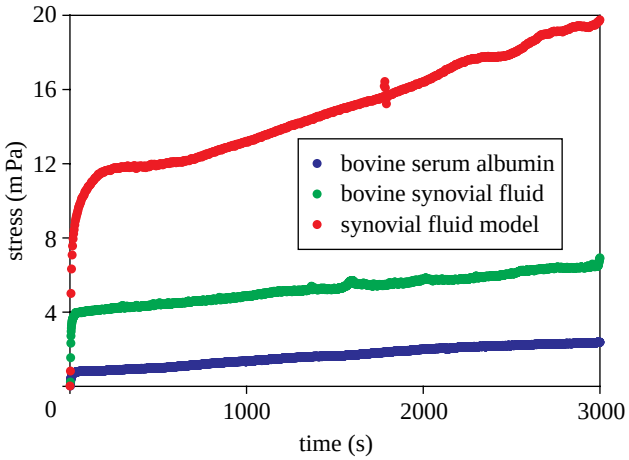

Figure 1. Time dependent stress in steady shear at 0.05 s^{-1} for the synovial fluid model (red or dark grey); bovine synovial fluid (green or light grey); and an 11 mg ml^{-1} solution of BSA in PBS (dark blue or black). All three tests immediately followed a preshear at 100 s^{-1} for 5 min and $t=0$ is shortly after the end of the preshear and the start of the application of 0.05 s^{-1}.

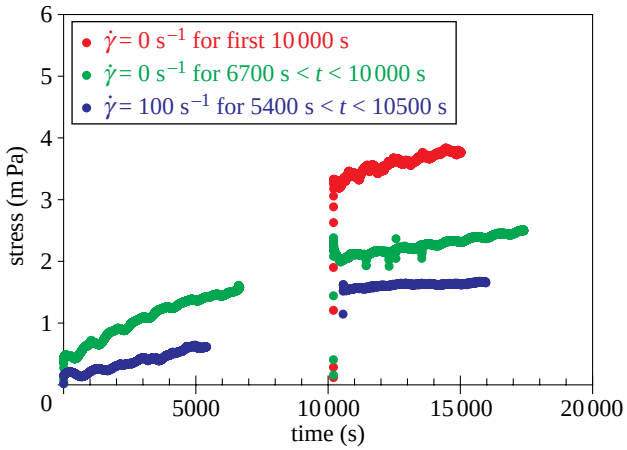

Figure 2. Time dependent stress in steady shear at 0.08 s^{-1} for 11 mg ml^{-1} BSA in PBS: with a 10 000 s delay (red or dark grey); with $\dot{\gamma}=0\ s^{-1}$ for 6700 $s<t<10\,000$ s (green or light grey); with $\dot{\gamma}=100\ s^{-1}$ for 5400 $s<t<10\,500$ s (dark blue or black). All three tests immediately followed a preshear at 100 s^{-1} for 5 min.

data at 25 °C, similar results are observed at 37 °C (Krause 2000; Oates 2002). The stress in low-viscosity polymer solutions under steady shear usually reaches its steady state value rapidly. In contrast, the rheopexy of figure 1 indicates that some type of structure is forming over time.

Figure 2 shows that the structure also builds under both quiescent conditions and under strong shear. Note that all samples for rheology were presheared at 100 s^{-1} for 5 min prior to measurements. The red points in figure 2 were obtained at $\dot{\gamma}=0.08\ s^{-1}$ after a 10 000 s delay (at $\dot{\gamma}=0\ s^{-1}$). The green points in figure 2 were obtained at $\dot{\gamma}=0.08\ s^{-1}$ with an interval between 6700 and 10 000 s where no shear was applied ($\dot{\gamma}=0\ s^{-1}$). The blue points in figure 2 were obtained at $\dot{\gamma}=0.08\ s^{-1}$ with an interval between 5400 and 10 500 s where $\dot{\gamma}=100\ s^{-1}$ was applied. While none of the data in figures 1 and 2 are fully reproducible, these data are representative of the time-dependent rheology we have observed. Figure 2 gives a good idea of the range of stresses observed at $\dot{\gamma}=0.08\ s^{-1}$ in different individual runs. The stress always builds with time at $\dot{\gamma}=0.08\ s^{-1}$ and always continues to build at $\dot{\gamma}=0\ s^{-1}$ (see red or dark grey and green or light grey points in figure 2) and $\dot{\gamma}=100\ s^{-1}$ (see dark blue or black points in figure 2), indicating structure formation. We have briefly explored other rotational rheometers and geometries with 11 mg ml^{-1} BSA in PBS, including cone and plate and a double concentric cylinder. Owing to torque resolution limitations, such measurements could only be made between 50 and 2000 s^{-1}. At such high shear rates, rheopexy is still observed but it is significantly less pronounced than at the low shear rates studied in figures 1 and 2 (and barely detectable at high shear rates). Structure builds fastest under quiescent conditions, and application of steady shear seems to retard structure growth, with slower rates of structure growth at higher shear rates.

What is the nature of this structure? Since rheopexy is observed for the synovial fluid model, rheopexy must be caused by the macromolecules in synovial fluid. The possibilities are: NaHA self-associations, protein aggregation, proteins binding to NaHA, or some combination of the three. NaHA self-associations are unlikely, as solutions of NaHA exhibit rheological behavior typical of ordinary, non-associating polymers (Milas *et al.* 1996; Krause *et al.* 2001). As long as the presence of the proteins does not disturb the local structure of NaHA (one proposed model for that structure is a tape-like helical structure (Scott 1989; De Smedt *et al.* 1993; Scott & Heatley 1999) the negative charges on NaHA ensure only a repulsive interaction with other NaHA chains.

Equilibrium dialysis suggests that there are only repulsive interactions between BSA and NaHA, at all concentrations studied (Oates 2002). The equilibrium protein concentration was higher in the surrounding 1 l buffer solution than in the NaHA solutions contained within the 5 ml dispodialyzer at all concentrations of NaHA and BSA explored. The concentration range of NaHA (0.3–6.0 mg ml^{-1}), included both dilute and semi-dilute concentrations (the overlap concentration $c^*=0.54$ mg ml^{-1}; Krause *et al.* 2001). In a control experiment, with only buffer in the dialysis tube, the BSA partitioned equally. Increasing the NaHA concentration resulted in reducing the amount of BSA in the NaHA solution. The results of this study are in qualitative agreement with the results of Ogston and Phelps (Ogston & Phelps 1960) and Dubin (Grymonpré *et al.* 2001). Binding between BSA and NaHA at physiological conditions is minimal. The interactions are dominated by electrostatic repulsion between BSA (net charge of $-15e$) and the anionic polysaccharide NaHA.

This leaves us with protein–protein interactions (Jaenicke & Helmreich 1972; Frieden & Nichol 1981; Fu 2004). Figures 1 and 2 show the rheopexy of an 11 mg ml^{-1} solution of BSA in PBS, indicating that proteins attract each other to form larger aggregates. The attraction leading to aggregation can be easily

understood from an electrostatic viewpoint. Even though each BSA molecule has a net charge of $-15e$, there are many positively charged amino acids that are primarily on one end of this globular protein. The radius of albumin is 3.5 nm (Nossal *et al.* 1986) and at pH = 7.4 the dipole moment is 1.3×10^{-27} Cm (Takashima 1965). This leads to an estimation (Gilson *et al.* 1985; Finkelstein & Ptitsyn 2002) of an attractive dipolar interaction energy of roughly $3kT$ for albumin, assuming a dielectric constant of 2 (meaning that $3kT$ is an upper bound for the real electrostatic interaction). The value of $3kT$ is similar to other estimates for BSA in the literature (Moon *et al.* 2000).

Although, figures 1 and 2 show that 11 mg ml^{-1} BSA solutions in PBS are rheopectic, with no steady state viscosity value, the apparent viscosity at any selected time shows a strong shear thinning for $0.01\ \mathrm{s}^{-1} < \dot{\gamma} < 100\ \mathrm{s}^{-1}$ (Oates 2002). This viscoelastic character is also consistent with the notion of BSA aggregation (non-aggregated 11 mg ml^{-1} BSA solutions would be expected to be Newtonian in this range of shear rates). Such shear thinning character has been reported for a variety of globular protein aqueous solutions (Tung 1978; Inoue & Matsumoto 1994; Matsumoto & Inoue 1996; Ikeda & Nishinari 2000, 2001*a*,*b*). Application of strong shear, ultrasonic treatment and sterile filtering of the albumin solutions did not qualitatively change the observed viscoelastic character of the BSA solutions, reflected in either the apparent shear thinning or the rheopexy (Oates 2002). Dialysis greatly reduces the concentrations of divalent cations in BSA but also did not appreciably change the viscoelastic character of the 11 mg ml^{-1} BSA solutions (Oates 2002).

SANS from solutions of BSA in D_2O (with PBS) show strong supporting evidence of protein aggregation in figure 3. The scattering intensity of BSA solutions at high wavevector was studied long ago (Ritland *et al.* 1950) and the form factor was calculated by Nossal, *et al.* (1986), shown as the solid curves in figure 3. The upturn in scattering intensity at low wavevectors is firm evidence of protein aggregation. The slope of that upturn determines the fractal dimension of the aggregates $D\cong2$, consistent with the fractal dimension of heat-set globular protein gels (Renard *et al.* 1999; Gosal & Ross-Murphy 2000). Indeed, $D=2.1$ was reported for heat-set albumin gels (Hagiwara *et al.* 1996). Values of fractal dimension of aggregating lysozyme (another globular protein) from light scattering have been reported to be primarily in the range $2.0<D<2.2$, with time dependent aggregation that depends on salt content (Umbach *et al.* 1998). The low wavevector slope of aggregating lysozyme observed with X-rays has also been reported to systematically change with salt concentration (Tardieu 1999).

4. DISCUSSION

The rheopexy of the three fluids in figures 1 and 2 is attributed to protein aggregation. Figure 3 shows that BSA aggregates under physiological conditions and it is likely that other globular proteins (globulins, etc.) may also be involved in the aggregates that form in synovial fluid and blood. A speculative illustration of the

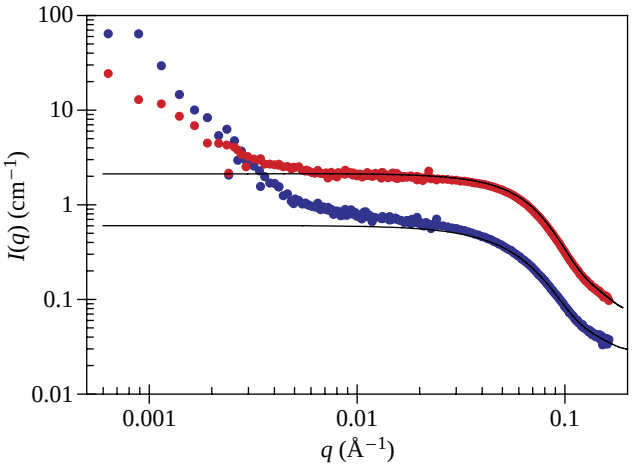

Figure 3. SANS data on BSA in D_2O with PBS at 25 °C. Data for two BSA concentrations are shown: typical for synovial fluid (11 mg ml^{-1}, dark blue or black) and typical for blood (44 mg ml^{-1}, red or grey). Solid curves are the form factors of BSA calculated by Nossal *et al.* (1986).

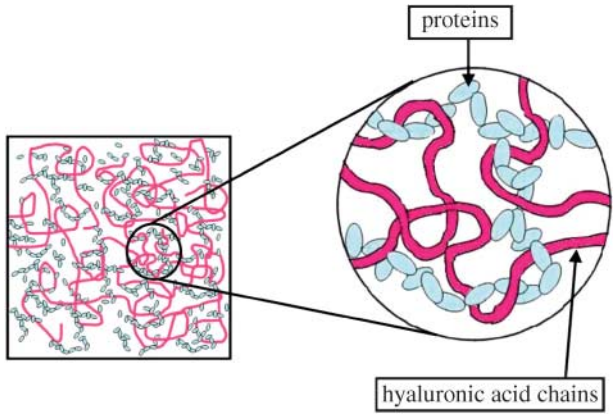

Figure 4. The proposed microstructure of synovial fluid: globular proteins (blue or light grey) aggregate to form a tenuous polymeric network and the long NaHA chains (red or dark grey) entangle with this network.

microstructure of synovial fluid is shown in figure 4, reflecting the fractal dimension $D=2$ as a random walk of aggregated proteins. By aggregating, the proteins create a tenuous network around the NaHA chains, creating more 'entanglements' in the solution. The fact that albumin has a net charge of $-15e$ and only sees an electrostatic attraction of order $3kT$ when in contact with a second albumin with perfect orientation, accounts for the slow rheopectic behavior seen in figures 1 and 2.

The presence of the high molecular weight NaHA chains in synovial fluid may enhance this attraction due to depletion flocculation (Renard *et al.* 1997; Kulkarni *et al.* 1999; Doublier *et al.* 2000; Tuinier & Brulet 2003). Indeed, NaHA depletion effects likely explain why the concentration of albumin in synovial fluid (11 mg ml^{-1}) is much lower than in blood (40–50 mg ml^{-1}). The idea of a 'volume exclusion' arising from the repulsion of the negatively charged proteins and anionic NaHA, effectively concentrating the proteins, is quite old (Laurent & Ogston 1963; Shaw 1976; Shaw & Schy 1977). Even neutral polymers such

as poly(ethylene oxide) are believed to concentrate proteins and encourage their aggregation (Nichol *et al.* 1981).

Weak association of the proteins is consistent with all data, including the ability of synovial fluid to flow and be pourable. The estimated binding energy of $3kT$ suggests that the fraction of non-aggregated albumin is $\exp(-3)=0.05$ and thus 95% of the albumin molecules are in the aggregated state. However, the weak $3kT$ attraction also suggests an association lifetime of order microseconds, which is too short to account for the observed rheopexy in figure 1. Most likely the proteins change their conformation (Bulone *et al.* 2001), exposing hydrophobic regions that enhance the interaction strength, and possibly involve inter-protein disulphide bond formation. Shear 'exposes the hydrophobic areas of proteins, causing aggregation' (Wang 2005). Such a conformational change apparently does not alter the α-helix content, as optical rotatory dispersion indicates no change in α-helix content for BSA during aggregation (Oates 2002). However, we cannot rule out the possibility of a conformational change that does not change the α-helix content (such as is seen in inclusion bodies; Finkelstein & Ptitsyn 2002).

This picture resolves the apparent contradiction between the conclusions of Dubin (Grymonpré *et al.* 2001) and those of Ogston and Stainer (Ogston & Stanier 1950, 1952). The illustration of figure 4 is consistent with Dubin's data, with no binding between NaHA and the proteins at pH = 7.4. The proteins, however, by aggregating around the NaHA chains, would 'lock up' a structure that has both protein and hyaluronic acid. This explains Ogston and Stanier's (1950, 1952) inability to separate the 'complex' into the individual components via non-destructive techniques. The structure suggested in figure 4 may be useful for interpreting the heterogeneous nature of the quaternary structure of albumin (Foster *et al.* 1965).

The protein aggregation of figures 1–3 suggests formation of a weak solid in BSA solutions in PBS. This solid-like character will help keep cartilage surfaces from coming into contact with one another in static conditions or at low shear rates. Indeed, joint stiffness following prolonged inactivity (such as sleep) may well be related to the build-up of the temporary protein network over time. This network behaves as a weak solid that is easily broken once the joint is forced to move. Solid-like rheology has been reported previously for albumin solutions, with a colloidal crystal structure proposed (Matsumoto & Inoue 1996; Ikeda & Nishinari 2000). At pH = 7.4 and 0.1 M ionic strength, there is insufficient repulsion to make a colloidal crystal structure (and the SANS data of figure 3 also do not support such a structure, as a colloidal crystal would have a peak in this range of q). A peak is seen in protein solutions at lower ionic strength, but it disappears when significant levels of salt are added (Renard *et al.* 1996). The osmotic pressure of BSA solutions with salt can be described using a strictly-repulsive interaction potential (Lin *et al.* 2002). However, globular proteins also phase separate on cooling and an interaction potential with a attractive well with depth of order $3kT$ is required to explain those observations (Malfois *et al.* 1996). Lysozyme has been modelled with a weak attractive interaction in computer simulations (Carlson *et al.* 2001). Aggregation has recently been suggested for other globular proteins (Piazza & Iacopini 2002; Piazza 2004) and may well be a general feature of globular protein solutions.

The solutions in figure 1 all show considerable viscoelasticity. When the shear rate is set to zero after extended shear in the Contraves viscometer, Newtonian liquids and even most polymer solutions of such low viscosity have their stress decay to zero within a few seconds (this time-scale is a property of the Contraves viscometer, not the sample). However, the protein-containing solutions show a much slower approach towards zero stress (Krause 2000), indicating enhanced elastic character in these liquids. This viscoelasticity is expected to give rise to normal stresses that act to push shearing surfaces apart, thereby aiding in lubrication. The rheological measurements performed in this study imitate the thick fluid film of a hydrodynamic lubricant, needed at high speeds and low loads (Booser 1994). However, the structural character implied by protein aggregation will also benefit boundary lubrication, necessary for low speeds and high normal loads, which occurs when the lubricant is adsorbed to cartilage surfaces, and its thickness is on the scale of nanometres (Booser 1994). The topic of thin film lubrication is an emerging area of tribology (Zhang 2005), which may prove invaluable to the study of joint lubrication. The idea that the weak solid character of synovial fluid affects lubrication of joints is consistent with Ogston & Stanier (1950, 1952), who suggested a connection between NaHA's ability to lubricate and its high viscosity at low shear rates with strong shear thinning to reduce drag at high shear rates.

5. CONCLUSIONS

Synovial fluid and BSA solutions in PBS are rheopectic: these fluids exhibit structure–building at sufficiently low shear rates (seen as stress increasing with time at low rates). This structure–building appears to also occur under quiescent conditions and the presence of NaHA increases the rate of structure–building (see figure 1). Rheological evidence for such structure–building in synovial fluid was reported in 1954 by ScottBlair and co-workers (ScottBlair *et al.* 1954), but has largely been ignored by the less-careful studies between then and now. SANS shows that BSA aggregates spontaneously under the physiological conditions found in synovial fluid and blood. Since NaHA solutions in PBS act as normal polymer solutions with no evidence of association (Krause *et al.* 2001) and equilibrium dialysis only suggests repulsion between NaHA and BSA (Oates 2002), we conclude that protein aggregation is responsible for the observed rheopexy. This aggregation strongly enhances the viscoelastic character of mammalian fluids such as synovial fluid and blood.

The rheopexy reported here is important, as it may be related to the superb lubrication properties of synovial fluid. Rheopectic behavior indicates a structure formation that could help explain joint stiffness after inactivity, as well as the desired fluid property of

increased elastic character that helps prevent joint surfaces from coming in contact. The connection between lubrication and rheopexy that we suggest here needs further investigation. Recent biolubrication studies (Tadmor *et al.* 2002; Zhu & Granick 2003; Benz *et al.* 2004) have focussed on thin film rheology tests of NaHA solutions, but proteins are essential for rheopectic behavior. Therefore, expansion of biolubrication studies to include proteins, either using bovine synovial fluid or our model synovial fluid, would enable the connection of rheopexy to boundary lubrication to be explored. By employing our synovial fluid model in biolubrication experiments, the role of protein aggregation could be directly tested, as our model contains no lubricin (a protein that has been suggested to be important for joint lubrication (Swann *et al.* 1985)).

REFERENCES

Anadere, I., Chmiel, H. & Laschner, W. 1979 *Biorheology* **16**, 179.

Balazs, E. A. 1970 In *Chemistry and molecular biology of the intercellular matrix* (ed. E. A. Balazs), vol. 3, pp. 1241–1253. New York: Academic Press.

Balazs, E. A. & Laurent, T. C. 1951 *J. Polym. Sci.* **6**, 665. (doi:10.1002/pol.1951.120060517)

Balazs, E. A. & Sundblad, L. 1959 *Acta Societatis Medicorum Upsaliensis* **64**, 137.

Balazs, E. A., Watson, D., Duff, I. F. & Roseman, S. 1967 *Arthr. Rheum.* **10**, 357.

Benz, M., Chen, N. & Israelachvili, J. 2004 *J. Biomed. Mater. Res.* **71A**, 6.

Berriaud, N., Milas, M. & Rinaudo, M. 1994 *Int. J. Biol. Macromol.* **16**, 137. (doi:10.1016/0141-8130(94)90040-X)

Bloch, B. & Dintenfass, L. 1963 *Aust. NZ J. Sur.* **33**, 108.

Bolen, J., Helmick, C. G., Sacks, J. J. & Langmaid, G. 2002 *Morb. Mortal. Wkly Rep.* **51**, 948.

Booser, E. R. (ed) 1994 *CRC handbook of lubrication and tribology Monitoring, materials, synthetic lubricants and applications*, vol. III. Boca Raton: CRC Press.

Bothner, H. & Wik, O. 1987 *Acta Otolaryn.* **442**, 25.

Bulone, D., Martorana, V. & Biagio, P. L. S. 2001 *Biophys. Chem.* **91**, 61. (doi:10.1016/S0301-4622(01)00155-7)

Carlsson, F., Malmsten, M. & Linse, P. 2001 *J. Phys. Chem. B* **105**, 12189. (doi:10.1021/jp012235i)

Certain commercial materials and equipment are identified in this paper in order to specify adequately the experimental procedure. In no case does such identification imply recommendation by the National Institute of Standards and Technology nor does it imply that the material or equipment identified is necessarily the best available for this purpose.

Chmiel, H. & Walitza, E. 1980 *On the rheology of blood and synovial fluids*. New York: Wiley.

Choi, S.-M. *et al.* 2000 *J. Appl. Cryst.* **33**, 793. (doi:10.1107/S0021889800099799)

Davies, D. V. & Palfrey, A. J. 1968 *J. Biomech.* **1**, 79. (doi:10.1016/0021-9290(68)90010-9)

De Smedt, S. C., Dekeyser, P., Ribitsch, V., Lauwers, A. & Demeester, J. 1993 *Biorheology* **30**, 31.

Doublier, J.-L., Garnier, C., Renard, D. & Sanchez, C. 2000 *Curr. Opin. Coll.* **5**, 202. (doi:10.1016/S1359-0294(00)00054-6)

Ferguson, J., Boyle, J. A., McSween, R. N. M. & Jasani, M. K. 1968 *Biorheology* **5**, 119.

Finkelstein, A. V. & Ptitsyn, O. B. 2002 *Protein physics*, Lecture 6. New York: Academic Press.

Foster, J. F., Sogami, M., Petersen, H. A. & Leonard, W. J. 1965 *J. Biol. Chem.* **240**, 2495.

Fouissac, E., Milas, M. & Rinaudo, M. 1993 *Macromolecules* **26**, 6945. (doi:10.1021/ma00077a036)

Frieden, C. & Nichol, L. W. 1981 *Protein–protein interactions*. New York: Wiley.

Fu, H. 2004 *Protein–protein interactions: methods and applications*. Totowa: Humana Press.

Gatej, I., Popa, M. & Rinaudo, M. 2005 *Biomacromolecules* **6**, 61. (doi:10.1021/bm040050m)

Gibbs, D. A., Merrill, E. W., Smith, K. A. & Balazs, E. A. 1968 *Biopolymers* **6**, 777. (doi:10.1002/bip.1968.360060603)

Gilson, M. K., Rashin, A., Fine, R. & Honig, B. 1985 *J. Mol. Biol.* **184**, 503. (doi:10.1016/0022-2836(85)90297-9)

Gomez, J. E. & Thurston, G. B. 1993 *Biorheology* **30**, 409.

Gosal, W. S. & Ross-Murphy, S. B. 2000 *Curr. Opin. Coll.* **5**, 188. (doi:10.1016/S1359-0294(00)00057-1)

Grymonpré, K. R., Staggemeier, B. A., Dubin, P. L. & Mattison, K. W. 2001 *Biomacromolecules* **2**, 422. (doi:10.1021/bm005656z)

Hagiwara, T., Kumagai, H. & Nakamura, K. 1996 *Biosci. Biotech. Biochem.* **60**, 1757.

Ikeda, S. & Nishinari, K. 2000 *Biomacromolecules* **1**, 757. (doi:10.1021/bm005587o)

Ikeda, S. & Nishinari, K. 2001*a* *Int. J. Biol. Macromol.* **28**, 315. (doi:10.1016/S0141-8130(01)00128-3)

Ikeda, S. & Nishinari, K. 2001*b* *Food Hydrocolloids* **15**, 401. (doi:10.1016/S0268-005X(01)00052-2)

Inoue, H. & Matsumoto, T. 1994 *J. Rheol.* **38**, 973. (doi:10.1122/1.550538)

Jaenicke, R. & Helmreich, E. (eds) 1972 *Protein–protein interactions*. Berlin: Springer-Verlag.

Johnson, R. S., Niedermeier, W. & Bobo, P. 1971 *Biorheology* **7**, 177.

Johnston, J. P. 1955 *Biochem. J.* **59**, 633.

Kobayashi, Y., Okamoto, A. & Nishinari, K. 1994 *Biorheology* **31**, 235.

Krause, W. E. 2000 Solution dynamics of synthetic and natural polyelectroltyes, Ph.D. thesis, The Pennsylvania State University.

Krause, W. E., Bellomo, E. G. & Colby, R. H. 2001 *Biomacromolecules* **2**, 65. (doi:10.1021/bm0055798)

Kulkarni, A. M., Chatterjee, A. P., Schweizer, K. S. & Zukoski, C. F. 1999 *Phys. Rev. Lett.* **83**, 4554. (doi:10.1103/PhysRevLett.83.4554)

Lapcik Jr, L., Lapcik, L., De Smedt, S., Demeester, J. & Chabrecek, P. 1998 *Chem. Rev.* **98**, 2663. (doi:10.1021/cr941199z)

Laurent, T. C. & Ogston, A. G. 1963 *Biochem. J.* **89**, 249.

Lin, Y.-Z., Li, Y.-G., Lu, J.-F. & Liu, Z.-P. 2002 *Mol. Phys.* **100**, 3251. (doi:10.1080/00268970210157903)

Linn, F. C. 1968 *J. Biomech.* **1**, 193. (doi:10.1016/0021-9290(68)90004-3)

Mabuchi, K., Tsukamoto, Y., Obara, T. & Yamaguchi, T. 1994 *J. Biomed. Mater. Res.* **28**, 865. (doi:10.1002/jbm.820280805)

Malfois, M., Bonnete, F., Belloni, L. & Tardieu, A. 1996 *J. Chem. Phys.* **105**, 3290. (doi:10.1063/1.471843)

Martin, R. B., Burr, D. B. & Sharkey, N. A. 1998 *Skeletal tissue mechanics*. New York: Springer-Verlag.

Matsudo, T., Ogawa, K. & Kokufuta, E. 2003 *Biomacromolecules* **4**, 728. (doi:10.1021/bm034033t)

Matsumoto, T. & Inoue, H. 1996 *Chem. Phys.* **207**, 167. (doi:10.1016/0301-0104(96)00036-5)

Mazzucco, D., McKinley, G., Scott, R. D. & Spector, M. 2002 *J. Orthopaedic Res.* **20**, 1157. (doi:10.1016/S0736-0266(02)00050-5)

McCutchen, C. W. 1978 In *The joints and synovial fluid* (ed. L. Sokoloff), ch. 10, pp. 437–483. New York: Academic Press.
Milas, M. *et al.* 2001 *Biopolymers* **59**, 191. (doi:10.1002/1097-0282(20011005)59:4<191::AID-BIP1018>3.0.CO;2-M)
Milas, M., Roure, I. & Berry, G. C. 1996 *J. Rheol.* **40**, 1155. (doi:10.1122/1.550778)
Mo, Y. & Nishinari, K. 2001 *Biorheology* **38**, 379.
Mo, Y., Takaya, T., Nishinari, K., Kubota, K. & Okamoto, A. 1999 *Biopolymers* **50**, 23. (doi:10.1002/(SICI)1097-0282(199907)50:1<23::AID-BIP3>3.0.CO;2-G)
Moon, Y. U., Curtis, R. A., Anderson, C. O., Blanch, H. W. & Prausnitz, J. M. 2000 *J. Sol. Chem.* **29**, 699. (doi:10.1023/A:1005112927213)
Mori, S., Naito, M. & Moriyama, S. 2002 *Int. Orthop.* **26**, 116. (doi:10.1007/s00264-002-0330-z)
Morris, E. R., Rees, D. A. & Welsh, E. J. 1980 *J. Mol. Biol.* **138**, 383. (doi:10.1016/0022-2836(80)90294-6)
Myers, R. R., Negami, S. & White, R. K. 1966 *Biorheology* **3**, 197.
Nichol, L. W., Ogston, A. G. & Wills, P. R. 1981 *FEBS Lett.* **126**, 18. (doi:10.1016/0014-5793(81)81022-8)
Nossal, R., Glinka, C. J. & Chen, S.-H. 1986 *Biopolymers* **25**, 1157. (doi:10.1002/bip.360250613)
Nuki, G. & Ferguson, J. 1971 *Rheol. Acta* **10**, 8. (doi:10.1007/BF01972470)
Oates, K. M. N. 2002 Biopolymer interactions in synovial fluid. Master's thesis, Pennsylvania State University.
Oates, K. M. N., Krause, W. E. & Colby, R. H. 2002 *Mater. Res. Soc. Symp. Proc.* **711**, 53.
Ogston, A. G. & Phelps, C. F. 1960 *Biochem. J.* **78**, 827.
Ogston, A. G. & Stanier, J. E. 1950 *Biochem. J.* **46**, 364.
Ogston, A. G. & Stanier, J. E. 1952 *Biochem. J.* **52**, 149.
O'Neill, P. L. & Stachowiak, G. W. 1996 *J. Orth. Rheum.* **9**, 222.
Piazza, R. 2004 *Curr. Opin. Coll. Int. Sci.* **8**, 515. (doi:10.1016/j.cocis.2004.01.008)
Piazza, R. & Iacopini, S. 2002 *Eur. Phys. J. E* **7**, 45. (doi:10.1007/s10189-002-8216-z)
Pigman, W., Gramling, E. & Holley, H. L. 1961 *Biochim. Biophys. Acta* **46**, 100. (doi:10.1016/0006-3002(61)90651-5)
Renard, D., Axelos, M. A. V., Boue, F. & Lefebvre, J. 1996 *J. Chim. Phys.* **93**, 998.
Renard, D., Boue, F. & Lefebvre, J. 1997 *Physica B* **234**, 289.
Renard, D., Lefebvre, J., Robert, P., Llamas, G. & Dufour, E. 1999 *Int. J. Biol. Macromol.* **26**, 35. (doi:10.1016/S0141-8130(99)00060-4)
Rha, C. K. & Pradipasena, P. 1986 *Viscosity of proteins, ch. 2.* Amsterdam: Elsevier.
Ritland, H. N., Kaesburg, P. & Beeman, W. W. 1950 *J. Chem. Phys.* **18**, 1237. (doi:10.1063/1.1747917)
Safari, M., Bjelle, A., Gudmundsson, M., Hoegfors, C. & Granhed, H. 1990 *Biorheology* **27**, 659.
Schurz, J. 1996 *J. Mat. Sci. Pure Appl. Chem.* **A33**, 1249.
Schurz, J. & Ribitsch, V. 1987 *Biorheology* **24**, 385.
Scott, J. E. 1989 *The biology of hyaluronan Ciba foundation symposium 143.* New York: Wiley pp. 6–20.
Scott, J. E. & Heatley, F. 1999 *Proc. Natl Acad. Sci. USA* **96**, 4850. (doi:10.1073/pnas.96.9.4850)
ScottBlair, G. W. 1974 *An introduction to biorheology.* Amsterdam: Elsevier.
ScottBlair, G. W., Williams, P. O., Fletcher, E. T. D. & Markham, R. L. 1954 *Biochem. J.* **56**, 504.
Shaw, M. 1976 *Biophys. J.* **16**, 43.
Shaw, M. & Schy, A. 1977 *Biophys. J.* **17**, 47.
Simkin, P. A. 1997 In *Arthritis and allied conditions* (ed. W. J. Koopman), ch. 9, pp. 193–205. Baltimore: Williams & Wilkins.
Sotiropoulou, M., Bokias, G. & Staikos, G. 2005 *Biomacromolecules* **6**, 1835. (doi:10.1021/bm050061v)
Sundblad, L. 1953 *Acta Soc. Med. Ups.* **58**, 113.
Swann, D. A. 1978 In *The joints and synovial fluid* (ed. L. Sokoloff), ch. 9, pp. 407–435. New York: Academic Press.
Swann, D. A., Silver, F. H., Slayter, H. S., Stafford, W. & Shore, E. 1985 *Biochem. J.* **225**, 195.
Tadmor, R., Chen, N. & Israelachvili, J. N. 2002 *J. Biomed. Mater. Res.* **61**, 514. (doi:10.1002/jbm.10215)
Takashima, S. 1965 *J. Phys. Chem.* **69**, 2281.
Tarasov, S., Kolubaev, A., Belyaev, S., Lerner, M. & Tepper, F. 2002 *Wear* **252**, 63. (doi:10.1016/S0043-1648(01)00860-2)
Tardieu, A. *et al.* 1999 *J. Cryst. Growth* **196**, 193. (doi:10.1016/S0022-0248(98)00828-8)
Tinico, I., Sauer, K., Wang, J. C. & Puglisi, J. D. 2002 *Physical chemistry: principles and applications in biological sciences*, 4th edn. London: Prentice Hall.
Tirtaatmadja, V., Boger, D. V. & Fraser, J. R. E. 1984 *Rheol. Acta* **23**, 311. (doi:10.1007/BF01332196)
Tuinier, R. & Brulet, A. 2003 *Biomacromolecules* **4**, 28. (doi:10.1021/bm0255544)
Tung, M. A. 1978 *J. Text. Stud.* **9**, 3.
Umbach, P., Georgalis, Y. & Saenger, W. 1998 *J. Am. Chem. Soc.* **120**, 2382. (doi:10.1021/ja964350q)
Wang, W. 2005 *Int. J. Pharm.* **289**, 1. (doi:10.1016/j.ijpharm.2004.11.014)
Welsh, E. J., Rees, D. A., Morris, E. R. & Madden, J. K. 1980 *J. Mol. Biol.* **138**, 375. (doi:10.1016/0022-2836(80)90293-4)
Xu, S., Yamanaka, J., Sato, S., Miyama, I. & Yonese, M. 2000 *Chem. Pharm. Bull.* **48**, 779.
Yanaki, T. & Yamaguchi, T. 1990 *Biopolymers* **30**, 415. (doi:10.1002/bip.360300319)
Yanaki, T. & Yamaguchi, M. 1994 *Chem. Pharm. Bull.* **42**, 1651.
Zhang, C. 2005 *Tribol. Int.* **38**, 443. (doi:10.1016/j.triboint.2004.10.011)
Zhu, Y. & Granick, S. 2003 *Macromolecules* **36**, 973. (doi:10.1021/ma025988r)