Concentrated solutions of salivary MUC5B mucin do not replicate the gel-forming properties of saliva

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We have developed a new approach to study the molecular organization of salivary mucus and salivary mucins using confocal fluorescence recovery after photobleaching (confocal-FRAP). MUC5B mucin, its reduced subunit and T-domains were prepared from saliva and fluorescently labelled. The translational self-diffusion coefficients were determined up to 3.6 mg/ml by confocal-FRAP. The results suggest that, in solutions of purified MUC5B mucin, at concentrations at which the hydrodynamic domains overlap, the intermolecular interactions are predominantly due to dynamic entanglements, and there was no evidence of specific self-association of MUC5B mucin, or of its subunits, or T-domains. The analysis of the salivary mucus gel also showed no specific interactions with the purified MUC5B components, but it was much less permeable than expected from its MUC5B content. The saliva was completely permeable to microspheres of 207 nm diameter, but

showed size-dependent effects on the diffusion of larger microspheres (499 nm and 711 nm diameter). From these analyses the salivary mucus was shown to be both permeable and dynamic, and with the characteristics of a semi-dilute transient network at physiological concentration. Comparison of the results from saliva and purified MUC5B mucin solutions showed that the network properties of saliva were equivalent to a solution of purified MUC5B mucin of 10–20 times higher concentration. This showed that saliva has additional structure and organization not present in the purified MUC5B mucin and suggests there are other interactions and/or components within saliva that combine with MUC5B to produce its complete properties.

Key words: confocal-FRAP, fluorescence recovery after photobleaching, mucus.

INTRODUCTION

Mucus is a viscoelastic gel that rheological studies have described as a network weakly cross-linked by non-covalent bonds [1,2]. Its rheology is determined by different factors such as its hydration [3], pH [4] and ion content [5–7]. Nevertheless, the properties of the mucus gel have been interpreted as being predominantly due to entanglement of the long, high-molecular-mass oligomeric mucins [3,8], but additional mechanisms of interaction have also been suggested. The interpretation of quasi-elastic light scattering, as well, as mechanical-spectroscopy studies, have suggested that interchain hydrophobic interactions [9], and carbohydrate–carbohydrate interactions between mucins [10–13] may stabilize the entangled gel. Electron-microscopic studies of human mixed saliva described a filamentous network structure with colloidal characteristics, which implied that a minimum concentration was required before a network formed [14]. Further electron-microscopic studies on dehydrated samples of cervical mucus have estimated an average mesh size of the network between 100 and 200 nm, and a similar estimate of the porosity of cervical mucus gel was obtained in exploratory fluorescence recovery after photobleaching (FRAP) studies [15].

Mucus coats the surfaces of the gastrointestinal and respiratory tracts as well as the oral cavity. In the mouth this is in the form of saliva, which is a dilute aqueous secretion that contains mucins, lipids and proteins (e.g. secretory IgA, lysozyme, amylase, statherin, histatin and proline-rich proteins) [16,17]. Saliva lubricates and protects the oral cavity and provides a non-

immune defence against pathogens [18,19]. The mucins are large secreted glycoproteins with many O-linked oligosaccharide chains, and human salivary mucins comprise two populations, MG1 and MG2, distinguished by their molecular masses. The MG1 fraction comprises predominantly the oligomeric *MUC5B* gene product and has a very high molecular mass $[(2–40) \times 10^3 \text{ kDa}]$ [20]. In contrast, the MG2 fraction is a product of the *MUC7* gene [21], which has a monomeric structure with a much lower molecular mass (120–150 kDa) [22]. The total concentration of mucin in saliva is approx. 200 $\mu\text{g/ml}$ [23]; however, specific determinations have reported the concentration of MUC5B and MUC7 as 233 $\mu\text{g/ml}$ and 133 $\mu\text{g/ml}$ respectively [24]. MUC4 mucin has been suggested to be present in salivary mucus, although no attempt was made to determine the amount present [25]. The compositional analysis and properties of the MG1 fraction from saliva suggest that MUC5B is the major mucin present [20,26]. There are other components associated with the salivary gel matrix, including secretory immunoglobulin A ('SIgA'), lactoferrin, lysozyme, MUC7 and agglutinin [27], and the cysteine-rich domains (Cys1, Cys2 and Cys8a) in MUC5B are potential binding sites for histatin and statherin [28].

The oligomeric MUC5B glycoproteins can be disassembled into constituent subunits [molecular mass $(2–3) \times 10^3 \text{ kDa}$] by reduction, and subsequent tryptic digestion yields high-molecular-mass glycopeptides (300–500 kDa) referred to here as 'T-domains' [29]. This mucin occurs in differently glycosylated low- and high-charge glycoforms [20,29]. In the oral cavity, the high charged populations are secreted from the palatal glands,

Abbreviations used: FA-HA, fluoresceinamine-hyaluronan; FRAP, fluorescence recovery after photobleaching; GdmCl, guanidinium chloride; PEG, poly(ethylene glycol).

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whereas the lower charged forms originate from the sublingual and submandibular glands [20]. The latter two glands secrete what has been described as 'insoluble' MUC5B assemblies that are found predominately in the gel phase of the secretion. However, in the whole saliva a greater proportion of the MUC5B mucin can be found in the sol phase in a lower-molecular-mass form [27].

FRAP provides a powerful approach to investigate the properties of macromolecules in concentrated solution [30]. It has been developed using a confocal microscope to study extracellular-matrix macromolecules such as hyaluronan [31–33], and enables molecular mobility to be determined in complex mixtures in the absence of concentration gradients and flow and shear forces [30]. In the present study we used confocal-FRAP to investigate intermolecular interaction, entanglement and gel formation in dilute and concentrated solutions of purified MUC5B mucin and its derived fragments. Moreover, we determined lateral tracer diffusion of MUC5B mucin probes in saliva at physiological concentrations. The tracer-diffusion measurements do not distinguish between gel formation, which involves stable, or semi-stable cross-links being formed between macromolecules, and entanglement, which occurs in all concentrated solutions of flexible polymers. In the present paper we therefore use the term 'network' to describe the intermolecular organization caused by gel formation or entanglement that impedes the free diffusion of other macromolecules in a concentration-dependent way. The network present in saliva was also characterized by measurement of the tracer diffusion of high-molecular-mass hyaluronan (830 kDa) and of rigid polystyrene microspheres of defined size. This permitted a direct comparison of the network formed in saliva with that in concentrated MUC5B mucin solutions and enabled the contribution of MUC5B mucin to the saliva network to be assessed.

EXPERIMENTAL

Saliva collection

Whole human saliva was collected fresh and centrifuged at 2700 *g* for 30 min at 4 °C to remove particulate matter. This native saliva was stored at 4 °C. It was used within 5 days and showed no significant change in properties in this time. For tracer-diffusion measurements with mucin probes, saliva was prepared as described above and was either diluted with PBS ('diluted' saliva) or concentrated by dialysis against Aquacide II (Calbiochem) ('concentrated' saliva). The content of MUC5B (see below) in diluted saliva was up to 0.11 mg/ml and in concentrated saliva was up to 0.45 mg/ml.

Mucin purification

The MUC5B mucins were extracted and purified as described previously [20]. Briefly, mucins were extracted from fresh saliva (overnight with gentle stirring at 4 °C) with 6 M guanidinium chloride (GdmCl) containing proteinase inhibitors (10 mM EDTA/10 mM *N*-ethylmaleimide/100 mM 6-aminohexanoic acid/5 mM benzamidine hydrochloride). After centrifugation (2700 *g*, 30 min, 4 °C), the supernatant was chromatographed on a size-exclusion column (1000 mm long × 52 mm diameter; Sepharose CL-2B; Amersham Pharmacia Biotech) eluted in 4 M GdmCl at a flow rate of 24 ml/h. MUC5B mucin fractions in the void volume of the column were purified by CsCl/4 M GdmCl density-gradient centrifugation (starting density 1.4 mg/ml) in a Beckman Ti45 rotor at 40000 rev./min for 65 h at 15 °C. The MUC5B mucin fractions were adjusted to 0.2 M GdmCl/CsCl (starting density of 1.5 mg/ml) for a second density-gradient

centrifugation in a Beckman Ti45 rotor at 40000 rev./min for 68 h at 15 °C. The MUC5B mucin fractions were pooled and concentrated by dialysis against poly(ethylene glycol) (PEG; 5%, w/v) to 0.5 mg/ml final concentration in 0.1 M carbonate/bicarbonate buffer, pH 9.0, and stored at 4 °C. This preparation was used for subsequent FITC labelling. Alternatively, MUC5B mucin fractions from the second density gradient were pooled and concentrated by dialysis against PEG (5%, w/v) in PBS to a final concentration of 1.2 mg/ml and used for the tracer-diffusion studies.

Fluorescent labelling of mucins and fragments

The purified MUC5B preparation was labelled with FITC (0.5 mg/ml) in 0.1 M carbonate/bicarbonate buffer, pH 9.0, for 48 h at 4 °C. The sample was run through a PD 10 Hi-Trap size-exclusion column (Amersham Pharmacia Biotech) in 4 M GdmCl, to remove unbound FITC. The excluded fraction was chromatographed on a Sepharose CL-2B column (1000 mm × 52 mm) eluted in 4 M GdmCl at a flow rate of 24 ml/h, with on-line monitoring of fluorescence using a spectrofluorimeter (Jasco, Tokyo, Japan) with excitation at 490 nm and emission at 520 nm. Fractions containing FITC-labelled MUC5B mucin (FITC-mucin) were pooled and divided in two. The first sample was concentrated by dialysis against PEG (5%, w/v) to 4 mg/ml in PBS (0.01 M phosphate/0.138 M NaCl/0.0027 M KCl) and stored at 4 °C for diffusion measurements.

The second FITC-mucin solution was reduced with 10 mM dithiothreitol in 6 M GdmCl/0.1 M Tris, pH 8.0. After incubation for 5 h at 25 °C, 25 mM iodoacetamide was added and the mixture was left in the dark for 30 min at 25 °C. The reduced and alkylated MUC5B mucin subunit (FITC-subunit) was chromatographed on a Sepharose CL-2B column with on-line fluorescence monitoring (as described above). Fractions containing the reduced FITC-subunit were pooled, dialysed and freeze-dried to constant mass and used for further experiments.

FITC-subunits were diluted in 0.1 M ammonium bicarbonate, pH 8.0, and trypsin (50 µg/mg of subunit) was added and incubated for 5 h at 37 °C. The MUC5B mucin digest was chromatographed on a Sepharose CL-2B column with fluorescence monitoring (as described above). Fractions containing FITC-T-domain were pooled, dialysed and freeze-dried to constant mass.

Determination of mucin concentration, molecular mass and radius of gyration

The concentration of MUC5B mucin in purified solutions and in saliva was determined by the following method. Samples of FITC-mucin solution and of saliva were reduced by dialysis against 6 M GdmCl/0.1 M Tris, pH 8.0, containing 10 mM dithiothreitol. The samples were chromatographed on a Sepharose CL-2B column (300 mm long × 10 mm diameter) eluted with 0.2 M NaCl/1 mM EDTA/0.05% sodium azide at a flow rate of 12 ml/h, and the solute concentration was determined using an in-line differential refractometer (Dawn DSP; Wyatt Technology, Santa Barbara, CA, U.S.A.). The mass of the mucin was calculated by integrating the void peak of the column and using a value for the refractive increment (dn/dc) of 0.16 ml/g (n is refractive index and c is concentration). These concentration measurements gave similar results, within 5%, to those determined using quantitative Western blotting employing a MUC5B mucin preparation from saliva as the standard [34].

The same system was used to determine the molecular mass and the radius of gyration of the purified MUC5B mucin, its

subunit and T-domains. Subunits and T-domains were prepared from intact mucins as described previously [29,35]. The intact mucins were chromatographed on a Sephacryl S-1000 column (300 mm × 10 mm) and the subunits and T-domains were chromatographed on a Superose 6 column (300 mm × 10 mm). Both columns were eluted with 4 M GdmCl at a flow rate of 12 ml/h, and the molecular mass and radius of gyration were determined using an in-line multiangle light-scattering photometer and a differential refractometer (Dawn DSP; Wyatt Technology). The molecular masses were calculated using a value for the refractive increment (dn/dc) in 4 M GdmCl of 0.130 ml/g.

Preparation of solutions for confocal-FRAP

For studies of the concentration-dependence of the self-diffusion of mucin and fragments, solutions of FITC-mucin, FITC-subunit and FITC-T-domain were diluted in PBS to 0.2–4.0 mg/ml. For tracer-diffusion measurements in saliva, solutions of FITC-mucin, FITC-subunit, FITC-T-domains, and fluorescein-amine-hyaluronan (FA-HA; 830 kDa) (each at 50 µg/ml) and FITC-BSA (12.5 µg/ml) were mixed with diluted or concentrated saliva in PBS. Similarly, fluorescent polystyrene microspheres (average diameter 207, 499 and 711 nm) were mixed (2%, w/v) with the diluted or concentrated saliva or purified whole mucin in PBS. Prior to mixing, all microspheres were coated for 12 h with 40 mg/ml BSA, washed and centrifuged (11 000 g, 2 min) to remove unbound BSA. All preparations were equilibrated overnight at 4 °C before confocal-FRAP experiments.

Diffusion measurements and data analysis

Confocal-FRAP experiments were carried out using a confocal laser microscope (MRC-1000; Bio-Rad, Hemel Hempstead, Herts., U.K.) with an upright epifluorescence microscope (Optiphot 2; Nikon, Tokyo, Japan) as described previously [31]. Generally, samples (40 µl) were sealed in a spherical-cavity microscope slide (diameter 12 mm, maximum depth 280 µm). For tracer-diffusion measurements using microspheres as probes, samples (10 µl) were sealed on a flat microscope slide. Lateral translational diffusion and tracer-diffusion coefficients were calculated from the time dependence of the plots of the second moment of the radially averaged distribution of bleached fluorophores [30]. The confocal-FRAP technique was previously shown to be non-destructive of macromolecular structure and to yield reproducible results comparable with other techniques for translational diffusion coefficients of proteins, proteoglycans and polysaccharides [31–33]. Five replicates were performed at 25 °C for each experiment.

RESULTS

Preparation of MUC5B mucin from saliva

MUC5B mucin was prepared from saliva by gel chromatography followed by two-step CsCl density-gradient centrifugation as described previously [20]. The mucin fractions from the second gradient were pooled (results not shown) and have been previously shown to comprise predominantly MUC5B mucin [20,26]. This fraction was used for fluorescent labelling.

In trial experiments it was found that a long incubation time with FITC was required to achieve sufficient labelling of the mucin. Routinely, 48 h at 4 °C with the reagent was used and the labelled FITC-mucin (MUC5B) was chromatographed on Sepharose CL-2B with on-line fluorescence monitoring (Figure 1a). The fluorescently labelled mucin was eluted in the void

Figure 1 Gel-filtration chromatography on Sepharose CL-2B of FITC-mucin and fragments in 4 M GdmCl

Chromatography of (a) FITC-mucin, (b) FITC-subunit, (c) FITC-T-domains was performed as described in the Experimental section. The column effluent was monitored on-line with a spectrofluorimeter (—■—■) and fractions (8 ml) were assayed for carbohydrate (—), after immobilization on nitrocellulose membrane, with the periodate–Schiff (PAS) reagent [36]. Fractions were pooled as shown by the horizontal bars.

volume of the column, similar to the unlabelled mucin. It showed no evidence of degradation by the labelling procedure and was well separated from unbound FITC.

Spectrofluorimetric measurements of the labelled mucin showed an average of 93 mol of FITC/mol. After reduction and alkylation, the FITC-mucin was eluted in a more included peak on the Sepharose CL-2B column (Figure 1b) and 10% of the total fluorescence was released and eluted in a peak next to the total column volume. Although there was some loss of fluorescence caused by the reduction process, the fluorescence of the pooled FITC-subunit was sufficient for further studies (9 mol of FITC/mol of subunit). Estimates of the lysine content of the subunit, using the deduced amino acid sequence [37–40], suggests that only about 10% of lysine residues were fluorescently

Figure 2 Self-diffusion measurements of FITC-labelled mucin and fragments

The concentration-dependence of (a) the relative diffusion of FITC-mucin (■), FITC-subunit (●) and FITC-T-domain (Δ) and (b) the lateral self-diffusion of FITC-mucin. All measurements were performed by confocal-FRAP in PBS at 25 °C. In (b) the continuous line shows a fit to eqn (3) by least-squares analysis. The error bars represent the S.D. of the calculated mean.

labelled. Trypsin digestion of the FITC-subunits released FITC-labelled T-domains, which were eluted in a more included position on the Sepharose CL-2B column (Figure 1c). A large fluorescent peak (95% of the total fluorescence) was eluted in the total column volume, which presumably contained FITC-labelled peptides liberated by the proteolytic digestion. The fluorescence of the FITC-T-domains was weak (0.5 mol of FITC/mol), but still sufficient for further studies.

Self-diffusion of FITC-mucin, FITC-subunit, and FITC-T-domain

The lateral self-diffusion coefficients were determined by confocal-FRAP of the FITC-whole mucin, FITC-subunit and FITC-T-domain preparations in PBS at concentrations from 0.2 to 4.0 mg/ml (Figure 2). From these measurements the equivalent hydrodynamic radius (R_H) was determined using the Stokes–Einstein approximation for the hydrodynamic behaviour of a sphere:

$$D = kT/6\pi\eta R_H \quad (1)$$

where D is the diffusion coefficient, k is Boltzman's constant; T is absolute temperature and η is the viscosity of PBS. The free diffusion coefficient (D^0) and equivalent diffusion and R_H were

Table 1 Physical properties of salivary MUC5B mucin and fragments

	$10^8 \times D^0$ ($\text{cm}^2 \cdot \text{s}^{-1}$)*	R_H (nm)*	$10^{-6} \times M_w$ (Da)†	R_g (nm)†
Whole mucin	2.49 ± 0.01	86	13.5	178
Subunit	8.28 ± 0.05	26	2.1	63
T-domain	17 ± 0.01	13	0.74	39

* Measurements were performed by confocal-FRAP on FITC-labelled mucin and derived components at 25 °C in PBS; the FITC-labelled fractions were of similar hydrodynamic size to corresponding unlabelled mucin fractions by size exclusion chromatography (Figure 1).

† Measurements were performed by light-scattering of unlabelled mucin fractions in 4 M GdmCl; M_w is weight-average molecular mass.

calculated using linear extrapolation to zero concentration for concentrations below 1 mg/ml (Table 1). Comparing the results of the whole mucin and the derived subfractions, there was a large increase in the D^0 values for the mucin and fragments as the mass decreased, which corresponded to a large decrease in hydrodynamic radius. The values are comparable with diffusion measurements performed in 6 M GdmCl with cervical mucin [35], which is also principally MUC5B [26].

At concentrations up to 3.6 mg/ml, the lateral diffusion coefficients of FITC-subunit and FITC-T-domain showed no detectable concentration-dependence at 25 °C (Figure 2a). There was thus no evidence for self-interaction of the FITC-T-domain and the FITC-subunit under these conditions. In contrast, the lateral diffusion coefficient of FITC-mucin was concentration-dependent above 1.0 mg/ml (Figure 2b). This result agrees with the domain overlap predicted by the Flory–Fox relationship:

$$c^* = 3M/N(4\pi R_g^3) \quad (2)$$

where c^* is the critical concentration, M is the molecular mass (13.5×10^3 kDa), N is Avogadro's number and R_g is the radius of gyration (178 nm) (Table 1). Above this critical concentration, the lateral self-diffusion coefficients decreased with concentration. The effects of domain overlap with increase in concentration on the diffusion coefficient are predicted by the universal scaling equation for polymers in semi-dilute solution [41]:

$$D = D^0 \exp(-ac^v) \quad (3)$$

where D is the measured polymer diffusion in the network, D^0 is the free diffusion coefficient, a and v are empirical constants and c is the concentration. The parameter a is a function of polymer size and describes the strength of polymer hydrodynamic interaction, and the deviation of v from unity arises from chain contraction at high concentration. The experimental results above the critical concentration were fitted by non-linear least-squares analysis to eqn (3) (Figure 2b), and gave $D^0 = 2.7 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$, $a = 0.48 \text{ ml/mg}$ and $v = 0.99$ ($R^2 = 0.96$).

Tracer diffusion in saliva of MUC5B mucin, subunit and T-domains

In order to investigate molecular interactions of MUC5B within saliva, we used the FITC-labelled mucin, subunit and T-domains as tracers within saliva itself. The saliva was either diluted or concentrated as described in the Experimental section so that we could manipulate the concentration of the MUC5B network; thus we have employed the concentration of MUC5B in saliva as our measure of the 'concentration' of saliva. At dilutions of saliva in which MUC5B concentrations were below 40 $\mu\text{g/ml}$, the diffusion coefficients of FITC-mucin, FITC-subunit and FITC-T-domain were equal to their free diffusion coefficients

Figure 3 Tracer diffusion studies in concentrated saliva

(a) The relative diffusion of FITC-labelled probes in a range of different concentrations of saliva: ■, FITC-mucin; ●, FITC-subunit; △, FITC-T-domain; ▼, FA-HA; ▽, FITC-BSA; ○, 207 nm microspheres. (b) The lateral diffusion of FITC-mucin and 207 nm diameter microspheres in a range of different concentrations of saliva. The lines show the fit to eqn (4) using least-squares analysis. Symbols are defined as in (a). The error bars represent the S.D. of the calculated mean. All measurements were performed by confocal-FRAP in PBS at 25 °C.

(Figure 3a). However, at increased concentrations of saliva the mobilities of the tracers began to fall, and this transition occurred at MUC5B concentrations above 46, 92 and 188 $\mu\text{g/ml}$, for the FITC-mucin, FITC-subunit and FITC-T-domain respectively. Above these concentrations, the relative mobility of the tracers decreased in relation to the molecular size. The decrease was the

biggest for FITC-mucin, smaller for the FITC-subunit and the smallest for the FITC-T-domain. Similar experiments were also carried out with FA-HA. The free diffusion of FA-HA ($6.04 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$) was in the same range, between the FITC-mucin value ($2.57 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$) and the FITC-subunit value ($8.28 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$). In saliva its diffusion became concentration-dependent at MUC5B concentrations of 131 $\mu\text{g/ml}$, but at higher concentrations the rate of decrease in diffusion of FA-HA was significantly lower than for the mucin tracers.

In the further analysis of these results it was apparent that only the intact FITC-mucin showed concentration-dependence of translational diffusion coefficients comparable with that predicted by the scaling equation for tracer diffusion in a semi-dilute polymer network, which is analogous to the equation of self-diffusion [41,42]:

$$D = D^0 \exp(-\beta c^v) \quad (4)$$

where D is the measured polymer diffusion in the network, D^0 is the free diffusion coefficient, and β and v are empirical constants. The parameter β is a linear function of the molecular size of the tracer (β/d is constant, where d is the diameter of the tracer) and describes the strength of the hydrodynamic interaction of the tracer, whereas v depends on the polymer network. The data for the FITC-mucin above the critical concentration were well-fitted by a non-linear least-squares analysis using eqn (4) (Figure 3b and Table 2), and there was no suggestion from the results that interactions other than entanglement were involved. In contrast, the data from FITC-subunit, FITC-T-domain and FA-HA did not fit well to eqn (4). This suggests that the model was inappropriate for these molecular tracers. This may be caused by the contraction of these tracers in the presence of other macromolecules, or networks, as has been demonstrated for the proteoglycan aggrecan and for HA [43].

Tracer diffusion in saliva of microspheres and FITC-BSA

The experiments on the diffusion of purified MUC5B mucin in saliva showed that the saliva matrix was permeable to macromolecules of quite large hydrodynamic size. In order to characterize the intermolecular network further, the permeability of three sizes of rigid fluorescent microspheres (207, 499 and 711 nm diameter) was determined in diluted saliva. In these experiments, microspheres were treated with BSA (see the Experimental section) to prevent non-specific interactions. The free diffusion coefficients of the BSA-coated microspheres were determined in PBS and R_H for each, calculated using the Stokes–Einstein approximation, was found to be within $\pm 10\%$ of the size reported by the manufacturer. This showed that the BSA coating had little effect on the lateral diffusion coefficients of the microspheres.

Table 2 Analysis of tracer diffusion in saliva

The tracer-diffusion values were fitted using a non-linear least-squares analysis to the scaling equation for tracer diffusion in semi-dilute polymer network (eqn 3) [41,42]. Critical concentration was estimated from the concentration at which diffusion became concentration-dependent for each tracer. β/d and v are characteristic of the network and are independent of the type of tracer. R^2 measures the fit of the experimental points to the theoretical curve. D^0 , β and v are taken as free parameters.

	Critical concn. (c^*) ($\mu\text{g/ml}$)	$10^9 \times \text{Lateral diffusion}$ (D^0) ($\text{cm}^2 \cdot \text{s}^{-1}$)	β (ml/mg)	$10^{-7} \times \beta/d$ ($\text{ml} \cdot \text{mg}^{-1} \cdot \text{m}^{-1}$)	v	R^2
Microspheres						
207 nm	98	21	1.87	0.9	0.79	0.97
499 nm	24	10.9	5.39	1.1	0.83	0.99
711 nm	12	6.4	7.91	1.1	0.82	0.91
FITC-mucin	46	27.5	1.84	1.1	0.82	0.98

Figure 4 Tracer diffusion studies in diluted saliva

(a) The relative diffusion of FITC-BSA (∇) and microspheres (207 nm, $\circ$; 499 nm, $\blacktriangle$; 711 nm, $\square$) over a range of concentrations of saliva. (b) The lateral diffusion of the 499 nm and 711 nm microspheres over a range of concentrations of saliva. The lines show the fit to eqn (4) using least-squares analysis. The error bars show the S.D. of the calculated mean. All measurements were performed by confocal-FRAP in PBS at 25 °C.

The lateral diffusion coefficient of FITC-BSA was also determined at different saliva concentrations and showed no evidence of interaction between BSA and saliva (Figure 3a and 4a).

The mobility of the 207 nm fluorescent microspheres showed no concentration-dependence in saliva, in which the MUC5B mucin content was up to 98 µg/ml (Figure 4a). However, the larger microspheres (499 and 711 nm) showed a marked concentration-dependence of their mobility in saliva above critical concentrations, at which the concentrations of MUC5B mucin were 24 µg/ml and 12 µg/ml respectively. The relative decrease in mobility was largest for the 711 nm microspheres. Experiments with more concentrated saliva and the 207 nm microspheres revealed concentration-dependence of their mobility in saliva in which the content of MUC5B was above 98 µg/ml (Figure 3a). It was noticeable that the decrease was much sharper for the rigid microspheres than for the more flexible mucin and HA probes. The data from the measurements with microspheres at above the critical concentration were well fitted by a non-linear least-squares analysis (Table 2) to eqn (4) (Figures 3b and 4b). Assuming the matrix is rigid, the parameters β and ν can be related to the correlation length of the network, ξ [44], which

Figure 5 Determination of the average mesh size of the salivary mucus network

The calculated average mesh size ξ determined from the tracer diffusion measurements with microspheres [207 nm ($\circ$), 499 nm ($\blacktriangle$) and 711 nm ($\square$)] over a range of different concentrations of saliva. The ξ values were calculated using eqn (5) and the fitted parameters from eqn (4) presented in Table 2.

describes the distance between chain-entanglement points [45]. The expression for ξ is:

$$\xi = (d/\beta)c^{-\nu} \quad (5)$$

where d is the diameter of the tracer. From the results the calculated values with the 499 and 711 nm microspheres were very similar over the same concentration range and were compatible with the results with the 207 nm microspheres at a higher MUC5B concentration (Figure 5).

The results using microspheres confirmed the results obtained with the mucin tracers and showed that the saliva network is permeable to even large macromolecules at physiological concentration. The analysis suggested that the saliva contained a dynamic network under a semi-dilute regime. Estimation of the average mesh size, ξ , over a range of MUC5B concentration showed good agreement of the results with microspheres of different size; however, this analysis may underestimate the size of the mesh, since the calculations involved assume that the network is rigid and immobile [46]. Microsphere diffusion experiments in solutions of purified MUC5B showed that the free diffusion of 711 nm microspheres was unaffected by MUC5B up to 320 µg/ml (results not shown). This showed MUC5B to be much more permeable than saliva at similar MUC5B concentrations, as predicted by the MUC5B self-diffusion and tracer-diffusion experiments.

DISCUSSION

The MUC5B mucin is the predominant high-molecular-mass oligomeric mucin in saliva [20] and in the present study its concentrated solution properties were investigated by confocal-FRAP. The mucin was purified and fluorescently labelled and it was also disassembled into its constituent subunits by reduction of disulphide bonds and then digested with trypsin to release high-molecular-mass glycopeptides or T-domains. Although lysine residues in the mucin modified with fluorescein may no longer be substrates for tryptic cleavage, the hydrodynamic size of the fluorescent T-domains are similar to unlabelled T-domains (results not shown). The fluorescent labelling, which modified about 10% of total lysine residues, did not therefore appear to affect significantly the size of the T-domain product. As a result

of the disassembly of the whole mucin, the number of fluorescent labelled sites decreased on the isolated fragments and the molar ratio of labelling fell from 90 in the whole mucin to 0.5 in the T-domain. The low extent of modification of the mucins and fragments is thus unlikely to affect their macromolecular properties.

The confocal-FRAP technique provides a new non-destructive approach to the characterization of mucus at physiological concentration, in the absence of flow and shear and under conditions that would favour intermolecular interactions. Using this method, the deduced free diffusion of MUC5B mucin and fragments are in general agreement with previous published values in 6 M GdmCl for the whole cervical mucin, its subunit and T-domain [35] (Table 1). Having established the free diffusion coefficients, it was interesting that the self-diffusion of FITC-subunit and the FITC-T-domain remained constant up to 3.6 mg/ml, which is ten times higher than the physiological concentration of MUC5B in saliva. This result is important, as it showed that there was no evidence of self-association occurring between MUC5B subunits, or amongst MUC5B T-domains, at these concentrations. These results, therefore, provide no evidence of hydrophobic interactions between protein segments, as have been suggested to mediate self-interaction for whole tracheo-bronchial mucins [9]. However, the mucin preparation used by Bromberg and Barr [9] may contain a mixture of different mucins and thus may have different properties. The results also show no evidence of carbohydrate-carbohydrate interactions between glycosylated regions at these concentrations, as has been proposed in other studies [10–13] with much higher concentrations of mucin, up to 35 mg/ml. Nevertheless, if these interactions occur between MUC5B mucin molecules in saliva at physiological concentrations, they would have to be very weak to evade detection in the confocal-FRAP experiments.

The analysis of self-diffusion of the intact MUC5B mucin shows that, below 1 mg/ml, there is little change with concentration, reflecting a dilute solution regime with no significant association between mucin molecules. At higher concentrations it shows the properties expected of a large polymer, with a concentration-dependent fall in lateral diffusion. This decrease occurred at the predicted critical concentration, c^* (1 mg/ml), for domain overlap, and the results fitted well with the hydrodynamic equation of polymers in semi-dilute solution predicting polymer domain overlap (eqn 3). These data suggest that domain overlap is the major cause for the decrease in mobility of the MUC5B mucin over this range of concentration. The concentration-dependence of the self-diffusion characteristics of the MUC5B mucin therefore show no detectable self-association arising from hydrophobic, oligosaccharide, or other interactions in dilute solution, and at higher concentrations the characteristics are those predicted by polymer entanglement. The absence of self-interaction between subunits and T-domains also supports the interpretation that the properties of purified intact MUC5B mucin at concentrations above 1 mg/ml are determined primarily by entanglement.

The mobility of FITC-mucin, FITC-subunit and FITC-T-domain were measured in saliva over a physiological range of concentrations. All the FITC-labelled MUC5B components showed a reduction in mobility above a critical concentration of saliva, but the mobilities were similar to free diffusion coefficients in diluted saliva, indicating that there was no detectable binding between MUC5B, its subunit or T-domain, with other macromolecular components in the saliva network. Furthermore, the changes in diffusion of the FITC-mucin in saliva at above the critical concentration, fitted with the hydrodynamic scaling model for tracer diffusion in a semi-dilute polymer solution (eqn 4). If

Figure 6 Comparison of the concentration dependence of the self-diffusion of MUC5B with its diffusion in concentrated saliva

Self-diffusion coefficient of MUC5B mucin (■) and tracer diffusion coefficient of FITC-mucin (◇) in concentrated saliva. The error bars show the S.D. of the calculated mean. All measurements were performed by confocal-FRAP in PBS at 25 °C.

the FITC-mucin is considered as its equivalent sphere ($R_H = 86$ nm, and thus $d = 172$ nm), the analysis gave similar values of β/d ($\beta/d \approx 1 \times 10^{-9}$) and ν ($\nu \approx 0.82$) for FITC-mucin and for the three different sizes of BSA-coated microsphere (Table 2). The similar β/d value suggests that the FITC-mucin moves in the saliva gel network in a way comparable with its equivalent sphere and it also implies that the FITC-mucin does not bind to the salivary network. The mobility of the FITC-mucin is therefore concluded to depend only on its hydrodynamic interactions with the network.

In contrast with the whole mucin, the tracer-diffusion behaviour of FITC-subunit, FITC-T-domain and FA-HA in diluted saliva could not be described by the universal scaling model [41]. The effect of increasing saliva concentration on the diffusion of these tracers was much less than would be predicted by this model. The results therefore, do not suggest any binding of these tracers to the saliva network, but may be better explained because these molecular tracers contract their domains as the saliva network concentration increases and they are therefore less affected by the increase in network concentration than a probe of fixed dimensions, such as a polystyrene microsphere.

As MUC5B mucin is the major high-molecular-mass oligomeric mucin in saliva, it would be expected to be the major component of a salivary network and its properties might be predicted to determine those of the mucus gel. However, the comparison between the diffusion of FITC-mucin in saliva and its self-diffusion over a range of MUC5B concentrations identified some major differences between the properties of purified MUC5B mucin and saliva (Figure 6). First, the critical concentration of MUC5B in saliva (46 μ g/ml), which affects the mobility of FITC-mucin tracer, is far lower than the critical concentration detected for domain overlap (1 mg/ml) in a purified MUC5B solution. Therefore the mobility of FITC-mucin is impeded by saliva at a concentration that is far below that corresponding to domain overlap in a concentrated solution of purified MUC5B mucin.

The results thus show that the physical properties of saliva are not reproduced in a concentrated solution of MUC5B mucin

alone. There is clearly another level of organization that makes saliva less permeable to both MUC5B mucin and to microspheres. The difference might be caused by the other elements, such as counter-ions [5–7] and pH [4], which may play an important role in the organization of the gel. In fresh saliva, ions, lipids, MUC7 mucin or other proteins may form cross-links within the MUC5B network, or/and they may participate in the formation of even larger assemblies of MUC5B mucin than those purified after 6 M GdmCl treatment. Further experiments are required to identify the basis of this additional level of organization.

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REFERENCES

- Madsen, F., Eberth, K. and Smart, J. D. (1998) A rheological examination of the mucoadhesive/mucus interaction: the effect of mucoadhesive type and concentration. *J. Control Release* **50**, 167–178.
- Bansil, R., Stanley, E. and LaMont, J. T. (1995) Mucin biophysics. *Annu. Rev. Physiol.* **57**, 635–657.
- Verdugo, P. (1990) Goblet cells secretion and mucogenesis. *Annu. Rev. Physiol.* **52**, 157–176.
- Phillips, J. G., James, S. L. and Lethem, M. I. (1997) Rheological properties and hydration of airway mucus. In *Airway Mucus: Basic Mechanisms and Clinical Perspectives* (Rogers, D. F. and Lethem, M. I., eds.), pp. 117–147. Birkhäuser, Basel, Boston and Berlin.
- Crowther, R. S., Marriott, C. and James, S. L. (1984) Cation induced changes in the rheological properties of purified mucus glycoprotein gels. *Biorheology* **21**, 253–263.
- Steiner, C. A., Litt, M. and Nossal, R. (1984) Effect of Ca^{2+} on the structure and rheology of canine tracheal mucin. *Biorheology* **21**, 235–252.
- Verdugo, P., Aitken, M., Langley, L. and Villalon, M. J. (1987) Molecular mechanism of product storage and release in mucin secretion. II. The role of extracellular Ca^{++} . *Biorheology* **24**, 625–633.
- Carlstedt, I., Sheehan, J. K., Corfield, A. P. and Gallagher, J. T. (1985) Mucus glycoproteins: a gel of a problem. *Essays Biochem.* **20**, 41–76.
- Bromberg, L. E. and Barr, D. P. (2000) Self-association of mucin. *Biomacromolecules* **1**, 325–354.
- McCullagh, C. M., Jamieson, A. M., Blackwell, J. and Gupta, R. (1995) Viscoelastic properties of human tracheobronchial mucin in aqueous solution. *Biopolymers* **35**, 149–159.
- Sellers, L. A., Allen, A., Morris, E. R. and Ross-Murphy, S. B. (1988) Mucus glycoprotein gels: Role of glycoprotein polymeric structure and carbohydrate side-chains in gel-formation. *Carbohydr. Res.* **178**, 93–110.
- Shogren, R., Jamieson, A. M., Blackwell, J., Cheng, P. W., Dearborn, D. G. and Boat, T. F. (1983) Solution properties of porcine submaxillary mucin. *Biopolymers* **22**, 1657–1675.
- Soby, L. M., Jamieson, A. M., Blackwell, J. and Jentoft, N. (1990) Viscoelastic properties of solutions of ovine submaxillary mucin. *Biopolymers* **29**, 1359–1366.
- Glantz, P. O., Wirth, S. M., Baeier, R. E. and Wirth, J. E. (1989) Electron microscopic studies of human mixed saliva. *Acta Odontol. Scand.* **47**, 7–15.
- Saltzman, W. M., Radomsky, M. L., Whaley, K. J. and Cone, R. A. (1994) Antibody diffusion in human cervical mucus. *Biophys. J.* **66**, 508–515.
- Beeley, J. A. (1993) Fascinating families of proteins: electrophoresis of human saliva. *Biochem. Soc. Trans.* **21**, 133–138.
- Iontcheva, I., Oppenheim, F. G. and Troxler, R. F. (1997) Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins. *J. Dent. Res.* **76**, 734–743.
- Wu, A. M., Csako, G. and Herp, A. (1994) Structure, biosynthesis, and function of salivary mucins. *Mol. Cell. Biochem.* **137**, 39–55.
- Veerman, E. C., Ligtenberg, A. J., Schenkels, L. C., Walgreen-Weterings, E. and Nieuw Amerongen, A. V. (1995) Binding of human high-molecular weight salivary mucins (MG1) to *Hemophilus parainfluenzae*. *J. Dent. Res.* **74**, 351–357.
- Thornton, D. J., Khan, N., Mehrotra, R., Howard, M., Veerman, E., Packer, N. H. and Sheehan, J. K. (1999) Salivary mucin MG1 is comprised almost entirely of different glycosylated forms of the MUC5B gene product. *Glycobiology* **9**, 293–302.
- Bobek, L. A., Tsai, H., Biesbrock, A. R. and Levine, M. J. (1993) Molecular cloning, sequence and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). *J. Biol. Chem.* **268**, 20563–20569.
- Mehrotra, R., Thornton, D. J. and Sheehan, J. K. (1998) Isolation and physical characterization of the MUC7 (MG2) mucin from saliva: evidence for self-association. *Biochem. J.* **334**, 415–422.
- Fox, P. C., Bodner, L., Tabak, L. A. and Levine, M. J. (1985) Quantitation of total human salivary mucins. *J. Dent. Res.* **64**, 327.
- Rayment, S. A., Liu, B., Offner, G. D., Oppenheim, F. G. and Troxler, R. F. (2000) Immunoquantification of human salivary mucins MG1 and MG2 in stimulated whole saliva: factors influencing mucin levels. *J. Dent. Res.* **79**, 1765–1772.
- Lui, B., Offner, G. D., Nunes, D. P., Oppenheim, F. G. and Troxler, R. F. (1998) MUC4 is a major component of salivary mucin MG1 secreted by the human submandibular gland. *Biochem. Biophys. Res. Commun.* **250**, 757–761.
- Wickström, C., Davies, J. R., Eriksen, G. V., Veerman, E. C. I. and Carlstedt, I. (1998) MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. *Biochem. J.* **334**, 685–693.
- Wickström, C., Christersson, C., Davies, J. R. and Carlstedt, I. (2000) Macromolecular organization of saliva: identification of 'insoluble' MUC5B assemblies and non-mucin proteins in the gel phase. *Biochem. J.* **351**, 421–428.
- Iontcheva, I., Oppenheim, F. G., Offner, G. D. and Troxler, R. F. (2000) Molecular mapping of statherin- and histatin-binding domains in human salivary mucin MG1 (MUC5B) by the yeast two-hybrid system. *J. Dent. Res.* **79**, 732–739.
- Thornton, D. J., Howard, M., Khan, N. and Sheehan, J. K. (1997) Identification of two glycoforms of the MUC5B mucin in human respiratory mucus: evidence for a cysteine-rich sequence repeated within the molecule. *J. Biol. Chem.* **272**, 9561–9566.
- Kubitscheck, U., Wedekind, P. and Peters, R. (1994) Lateral diffusion measurement at high spatial resolution by scanning microphotolysis in a confocal microscope. *Biophys. J.* **67**, 948–956.
- Gribbon, P. and Hardingham, T. E. (1998) Macromolecular diffusion of biological polymers measured by confocal fluorescence recovery after photobleaching. *Biophys. J.* **75**, 1032–1039.
- Gribbon, P., Heng, B. C. and Hardingham, T. E. (1999) The molecular basis of the solution properties of hyaluronan investigated by confocal fluorescence recovery after photobleaching. *Biophys. J.* **77**, 2210–2216.
- Gribbon, P., Heng, B. C. and Hardingham, T. E. (2000) The analysis of intermolecular interactions in concentrated hyaluronan solutions suggest no evidence for chain-chain association. *Biochem. J.* **350**, 329–335.
- Kirkham, S., Sheehan, J. K., Knight, D., Richardson, P. S. and Thornton, D. J. (2002) Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. *Biochem. J.*, in the press.
- Sheehan, J. K. and Carlstedt, I. (1984) Hydrodynamic properties of human cervical-mucus glycoproteins in 6 M guanidinium chloride. *Biochem. J.* **217**, 93–101.
- Thornton, D. J., Holmes, D. F., Sheehan, J. K. and Carlstedt, I. (1989) Quantification of mucus glycoproteins blotted onto nitrocellulose membranes. *Anal. Biochem.* **182**, 160–164.
- Buisine, M. P., Desseyn, J. L., Porchet, N., Degand, P., Laine, A. and Aubert, J. P. (1998) Genomic organization of the 3'-region of the human *MUC5AC* mucin gene: additional evidence for a common ancestral gene for the 11p15.5 mucin gene family. *Biochem. J.* **332**, 729–738.
- Desseyn, J. L., Guyonnet-Dupérat, V., Porchet, N., Aubert, J. P. and Laine, A. (1997) Human mucin gene *MUC5B*, the 10.7-kb large exon encodes various alternate subdomains resulting in a super-repeat. *J. Biol. Chem.* **272**, 3168–3178.
- Desseyn, J. L., Aubert, J. P., Van Seuningen, I., Porchet, N. and Laine, A. (1997) Genomic organization of the 3' region of the human mucin gene *MUC5B*. *J. Biol. Chem.* **272**, 16873–16883.
- Desseyn, J. L., Buisine, M. P., Porchet, N., Aubert, J. P. and Laine, A. (1998) Genomic organization of the human mucin gene *MUC5B*. *J. Biol. Chem.* **273**, 30157–30164.
- Phillies, G. D. J. (1989) The hydrodynamic scaling model for polymer self-diffusion. *J. Phys. Chem.* **93**, 5029–5039.
- Langevin, D. and Rondelez, F. (1978) Sedimentation of large colloidal particles through semi-dilute polymer solutions. *Polymer* **19**, 875–882.
- Harper, G. S. and Preston, B. N. (1987) Molecular shrinkage of proteoglycans. *J. Biol. Chem.* **262**, 8088–8095.
- De Smedt, S. C., Lauwers, A., Demeester, J., Engelborghs, Y., De Mey, G. and Du, M. (1994) Structural information on hyaluronic acid solutions as studied by probe diffusion experiments. *Macromolecules* **27**, 141–146.
- De Gennes, P. G. (1979) *Scaling Concepts in Polymer Physics*, Cornell University Press, Ithaca, NY.
- Kluitjans, S. G. J. M., Kocndrink, G. H. and Philipse, A. P. (2000) Self-diffusion and sedimentation of tracers spheres in (semi) dilute dispersions of rigid colloidal rods. *Phys. Rev. E* **61**, 626–636.

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