Chapter 2

Gel-Forming and Cell-Associated Mucins: Preparation for Structural and Functional Studies

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Abstract

Secreted and transmembrane mucins are important components of innate defence at the body's mucosal surfaces. The secreted mucins are large, polymeric glycoproteins, which are largely responsible for the gel-like properties of mucus secretions. The cell-tethered mucins, however, are monomeric but are typically composed of two subunits, a larger extracellular subunit which is heavily glycosylated while the smaller more sparsely glycosylated subunit has a short extracellular region, a single-pass transmembrane domain, and a cytoplasmic tail. These two families of mucins represent high-molecular-weight glycoproteins containing serine and threonine-rich domains that are the attachment sites for large numbers of O-glycans. The high-M_r and high sugar content have been exploited for the separation of mucins from the majority of components in mucus secretions. In this chapter, we describe current and well-established methods (caesium chloride density-gradient centrifugation, gel-filtration and anion-exchange chromatography, and agarose gel electrophoresis) for the extraction and purification of gel-forming and cell-surface mucins which can subsequently be used for a variety of structural and functional studies.

Key words: Density-gradient centrifugation, Mucin extraction, Mucin purification, Guanidinium chloride, Gel-filtration chromatography, Anion-exchange chromatography, Agarose gel electrophoresis, Mucin

1. Introduction

Mucins are important components of innate defence at the body's mucosal surfaces (1). This family of multifunctional glycoproteins comprises transmembrane (cell surface) and secreted mucins. The transmembrane mucins are localised at the apical surface of epithelial cells and are part of the carbohydrate-rich glycocalyx within which they function to protect and "sense" the immediate environment at the epithelial cell surface. By providing the structural framework of mucus, the secreted gel-forming mucins form a dynamic protective barrier that lies above the glycocalyx. In normal physiology,

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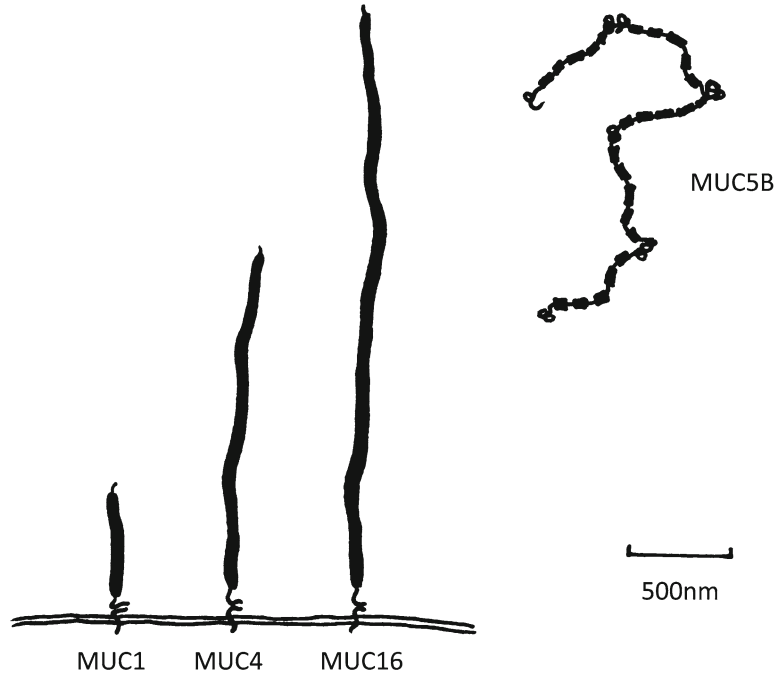


Fig. 1. Schematic drawing of MUC1, MUC4, MUC16, and MUC5B mucins. MUC1, MUC4, and MUC16 are depicted as cell-associated mucins with a putative cleavage site close to the cell membrane. The longest reported allele is shown for each mucin species. The polymeric gel-forming mucin comprises five MUC5B subunits. The four mucins are drawn to scale relative to each other with the lengths of the glycosylated domains (*thick lines*) estimated using the assumption that the length/amino acid is 0.25 nm in this part of the molecule.

these two mucin-rich environments act in concert to protect the mucosal surfaces from chemical, physical, and biological challenge.

The secreted mucins (MUC2, MUC5AC, MUC5B, MUC6, MUC7, and MUC19), with the exception of MUC7, are large, polymeric glycoproteins that are largely responsible for the gel-like properties of mucus secretions (2). The polymeric gel-forming mucins are assembled via disulphide linkages at the C- and N-termini of their constituent monomers (subunits), each monomer comprising a heavily glycosylated central region that is interspersed with cysteine-rich domains and flanked by cysteine-rich terminal domains (Fig. 1). In contrast, the cell-surface mucins (e.g. MUC1, MUC4, and MUC16) are monomeric but are typically composed of two subunits, the larger subunit is wholly extracellular and heavily glycosylated while the smaller more sparsely glycosylated subunit consists of a short extracellular region, a single-pass transmembrane domain, and a cytoplasmic tail (3) (Fig. 1). While these two families of mucins have quite different structural organisation, they do share common features; both are high-molecular-weight glycoproteins (0.25–50 MDa) with large, often tandemly repetitive sections of their polypeptides, rich in serine and threonine that are the

attachment sites for O-glycans. This arrangement results in mucins having a high proportion of their mass as carbohydrate (30–80% of weight). These two features (high-M_r and high sugar content) have been exploited for the separation of mucins from the majority of proteins and other glycoproteins.

In this chapter, we describe current and well-established methods for the extraction and purification of secreted, gel-forming and cell-surface mucins. These protocols can be used to isolate mucins as the starting point for subsequent structural and functional studies, for example: analysis of mucin size distribution and morphology by electron microscopy, mass spectrometry-based analysis of the mucin polypeptide (identification of gene products or proteolytic processing) and mucin glycans, reagents for studying bacterial binding and drug interactions, or as a means of identifying mucin-binding proteins to better understand how the mucus layer is organised. Of course, the goals of the subsequent studies dictate the solvent conditions of extraction and purification (i.e. whether chaotropic denaturing agents can be used or if it is necessary for the mucins to be in a "native" state) (see Note 1).

2. Materials

2.1. Extraction of Mucins with Chaotropic Agents

1. Guanidinium chloride solution
   During each step in the mucin preparation, it is worth ensuring that the quality of the chemicals used is of sufficiently high quality not to interfere with subsequent analyses. Guanidinium chloride can either be purchased as ultrapure grade, which needs no further treatment, or as practical grade in which case a purification step is required. The use of the latter is considerably less expensive and is worth the extra work if large amounts are to be used. We prepare a *stock* solution with a molarity of approx. 7.5 M and use it to make all the necessary guanidinium chloride solutions.
   (a) Dissolve 765 g of guanidinium chloride in 1 L of distilled water and stir constantly.
   (b) Add 10 g of activated charcoal and stir overnight.
   (c) Filter solution through double-filter paper to remove the bulk of the charcoal.
   (d) To remove remaining charcoal, filter solution through an Amicon PM10 filter (Millipore, Billerica, MA) or equivalent using an ultrafiltration cell. The speed and capacity of filtration can be increased by using a Diaflow system.
   (e) Measure the density of the solution by weighing a known volume and calculating the molarity of the guanidinium chloride stock solution (see Note 2).

2. Solutions for mucin extraction
   (a) 6 M guanidinium chloride extraction buffer: 6 M guanidinium chloride (from stock), 5 mM EDTA, 10 mM sodium phosphate buffer, pH 6.5 (adjusted with NaOH). This solution can be stored at room temperature but should be cooled to 4°C for use. Immediately prior to extraction, add *N*-ethylmaleimide (NEM) and diisopropyl phosphofluoridate (DPF) or phenylmethylsulphonylfluoridate (PMSF) (see Note 3) to the solution to give concentrations of 5 mM NEM and 1 mM DFP or 0.1 mM PMSF.
   (b) Phosphate-buffered saline (PBS) containing protease inhibitors: 0.2 M sodium chloride, 10 mM EDTA, 10 mM NEM, 2 mM DFP, 10 mM sodium phosphate buffer, pH 7.4 (adjusted with NaOH).
   (c) 6 M guanidinium chloride reduction buffer: 6 M guanidinium chloride (from stock), 5 mM EDTA, 0.1 M Tris–HCl buffer, pH 8.0 (adjusted with HCl). This solution can be stored at room temperature (see Note 4).

2.2. Extraction of Mucins with Non-chaotrophic Agents

For some purposes, it may be necessary to prepare "native" mucins under non-denaturing or associative conditions. Under these conditions, the mucus gel is not completely solubilised and only those mucins that are easily released from the complex are present in solution. Thus, the risk that some mucin populations may be selectively lost in subsequent purification and centrifugation steps should be borne in mind.

1. 0.2 M sodium chloride solution (pH 7).
2. 10 mM Tris buffer, pH 8.0, containing detergent (0.5% (v/v) Triton X-100 or 0.5% (w/v) sodium deoxycholate).

2.3. Isopycnic Density-Gradient Centrifugation

Purification of mucins from complex secretions and tissues often requires a two-step isopycnic density-gradient centrifugation procedure using CsCl to remove proteins (first step) and nucleic acids (second step). Caesium chloride can be purchased as ultrapure grade, but we order small samples of less pure caesium chloride from several companies and test them to ensure that they give clear solutions with low absorbance at 280 nm and do not give spurious reactions in our assays before purchasing larger batches (see Note 5).

1. Beckman Quick seal polyallomer centrifuge tubes (Beckman Instruments, Palo Alto, CA) or equivalent.
2. 6 M guanidinium chloride extraction buffer, pH 6.5.
3. Sodium phosphate buffer: 10 mM sodium di-hydrogen phosphate, pH 6.5 (adjusted with NaOH).
4. 0.5 M guanidinium chloride buffer: 0.5 M guanidinium chloride (from stock), 5 mM EDTA, 10 mM sodium phosphate buffer, pH 6.5 (adjusted with NaOH).

2.4. Gel Chromatography

After CsCl density-gradient centrifugation, gel-filtration chromatography can be employed to separate high-M_r mucins from smaller proteins and glycoproteins. We use a variety of separation media, including Sephacryl S-1000, Sephacryl S-500HR Sepharose CL-2B, and Superose 6 gels (GE Healthcare Life Sciences) (see Notes 6 and 7).

1. Denaturing conditions
   (a) 4 M guanidinium chloride buffer: 4 M guanidinium chloride (from stock), 10 mM sodium phosphate buffer, pH 7.0 (adjusted with NaOH). This solution can be stored at room temperature.
2. Non-denaturing conditions
   (b) 0.15 M NaCl solution.

2.5. Anion-Exchange Chromatography

In our laboratories, we use Mono Q HR 5/5 or Resource Q columns (GE Healthcare Life Sciences) and eluants based on a piperazine buffer system with lithium perchlorate as the elution salt (see Note 8).

1. Buffer A: 10 mM piperazine/perchlorate buffer, pH 5.0 (adjusted with perchloric acid).
2. Buffer B: 0.25–0.4 M $LiClO_4$, 10 mM piperazine/perchlorate buffer, pH 5.0 (adjusted with perchloric acid).
3. Buffer C: 0.1% (w/v) 3-((3-cholamidopropyl)dimethylammonio)-1-propane sulfonate (CHAPS) in 6 M urea, 10 mM piperazine/perchlorate buffer, pH 5.0 (adjusted with perchloric acid).
4. Buffer D: 0.1% (w/v) CHAPS in 6 M urea, 0.25–0.4 M $LiClO_4$, 10 mM piperazine/perchlorate buffer, pH 5.0 (adjusted with perchloric acid).

2.6. Agarose Gel Electrophoresis

In addition to the preparative techniques above, analytical separation can be achieved by agarose gel electrophoresis and the separation monitored by lectin, immunochemical, or histochemical staining after transfer of the protein to membranes (for detection methods, see Chapter 3). This technique can be applied to "raw" mucus samples, although care must be taken to ensure that samples are protected from degradation. This separation, which works on the basis of inherent negative charge on the sialic acid and sulphate groups on the mucin glycans (4), can be used to separate different mucins (5) as well as glycosylated variants of the same mucin (6). Furthermore, this technique allows separation of mature and precursor forms of the mucins.

1. Horizontal gel electrophoresis apparatus for 25 × 15-cm or 15 × 15-cm gels (we are using Bio-RAD Subcell apparatus).
2. Ultrapure agarose.

3. Running buffer: 40 mM Tris–acetate, 1 mM sodium EDTA, pH 8.0, containing 0.1% (w/v) sodium dodecyl sulphate (SDS).
4. Loading buffer: 10× running buffer containing 50% (v/v) glycerol and 0.002% (w/v) bromophenol blue.
5. Transfer buffer: 0.6 M NaCl, 60 mM sodium citrate.

3. Methods

3.1. Mucin Extraction

3.1.1. Extraction of Gel-Forming Mucins from Mucus Secretions

For some purposes, it may be necessary to solubilise a viscoelastic mucus gel in order to extract the mucins. In this case, the protocol below, for extraction under denaturing conditions, should be followed.

Denaturing Conditions

1. Thaw secretions on ice, if necessary, mix with an equal volume of ice-cold PBS containing protease inhibitors and stir gently at 4°C for 1 h.
2. Centrifuge secretions (23,000 × *g*, 4°C, 45 min).
3. Pour off the supernatant which represents the sol phase.
4. Add at least five volumes of 6 M guanidinium chloride extraction buffer to the pellet (which represents the gel phase) and stir gently overnight at 4°C. If the samples contain material that is difficult to disperse, they can be suspended using two or three strokes in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) with a loose pestle.
5. Centrifuge secretions (23,000 × *g*, 4°C, 45 min).
6. Pour off the supernatant. This corresponds to the "guanidinium chloride soluble" gel-phase mucins.
7. If necessary, repeat steps 4–6 two more times and pool the extracts.
8. Add 6 M guanidinium chloride reduction buffer containing 10 mM dithiothreitol (DTT) to the pellet (corresponding to the "guanidinium chloride insoluble" mucins) (see Note 9) and incubate for 5 h at 37°C.
9. Add iodoacetamide (IAA) to give a 25 mM solution, and incubate overnight in the dark at room temperature. This sample corresponds to the reduced/alkylated guanidinium chloride "insoluble mucin complex".

Non-denaturing (Associative) Conditions

In cases where preparation of "native" mucins under non-denaturing (associative) conditions is required, the following protocol should be used (see Note 10).

1. Thaw secretions on ice, if necessary, mix with an equal volume of ice-cold PBS containing protease inhibitors, and stir gently at 4°C for 1 h.

2. Centrifuge secretions (4,400×*g*, 4°C, 30 min).
3. Pour off and retain the supernatant for further purification.

3.1.2. Extraction of Gel-Forming Mucins from Tissue Samples

Since tissue extracts contain high levels of cell debris and extracellular matrix fragments, as well as proteolyic enzymes, we always prepare mucins from this source under denaturing conditions. If mucins are to be prepared from the surface epithelium and submucosa separately, begin at step 1, whereas if they are to be extracted from the whole tissue piece, begin at step 4.

1. Drench tissue pieces in 10 mM sodium phosphate buffer, pH 6.5, containing 1 mM DFP or 0.1 mM PMSF and allow to thaw if frozen.
2. Scrape the surface epithelium away from the underlying mucosa using a blunt instrument or a glass microscope slide.
3. Place the epithelial scrapings in at least five volumes of ice-cold 6 M guanidinium chloride extraction buffer and disperse with two or three strokes in a Dounce homogenizer with a loose pestle.
4. Cut the submucosal tissue (or whole tissue piece) into small pieces using scissors or a scalpel and submerge in liquid nitrogen. Pulverize or grind the tissue (we use a Retch tissue pulverizer, Retsch, Haan, Germany) for this purpose.
5. Mix the powdered tissue with at least five volumes of ice-cold 6 M guanidinium chloride extraction buffer and disperse with two or three strokes in a Dounce homogenizer with a loose pestle.
6. Gently stir samples overnight at 4°C.
7. Centrifuge secretions (23,000×*g*, 4°C, 45 min).
8. Pour off the supernatant corresponding to the “guanidinium chloride soluble” mucins.
9. Repeat steps 5–8 two more times if necessary and pool the extracts.
10. Add 6 M guanidinium chloride reduction buffer containing 10 mM DTT to the extraction residue and incubate for 5 h at 37°C.
11. Add IAA to give a 25 mM solution, and incubate overnight in the dark at room temperature.
12. Pour off the supernatant corresponding to the reduced/alkylated guanidinium chloride “insoluble mucin complex”.

3.1.3. Extraction of Gel-Forming Mucins from Mucin-Producing Cell Cultures

Mucin-producing cell lines and primary cell cultures represent a valuable complement to tissues and secretions for studies of the structure and function of these molecules. Many tumour cell lines overexpress cell-associated mucins making them good tools for investigation of, for instance, the biosynthesis and receptor functions

of these molecules. Cells derived from intestinal and airway tumours or normal tissue have proved very useful for studies of biosynthesis and assembly of the large, gel-forming polymeric mucin species as well as providing a ready source of relatively "clean" secretions.

1. Cell culture medium
   Mucins may be extracted from aspirated cell culture medium under denaturing or non-denaturing conditions using the appropriate protocol given for extraction from mucus secretions (Subheading 3.1.1 or 3.1.2).
2. Cell layers
   Extraction of gel-forming mucins from the cell layer of a culture can be carried out as follows.
   (a) After removing the culture medium by aspiration, wash the cell layer with PBS.
   (b) Separate the cell layer from the substrate using, for instance, a cell scraper, and add a 0.5–1 mL of ice-cold 6 M guanidinium chloride extraction buffer per cm^2. Disperse the cells using a Pasteur pipette.
   (c) Gently stir the sample overnight at 4°C.
   (d) Centrifuge secretions (23,000 × *g*, 4°C, 45 min).
   (e) Pour off the supernatant corresponding to the "guanidinium chloride soluble" mucins.
   (f) Repeat steps 2–5 two more times if necessary and pool the extracts.
   (g) Add 6 M guanidinium chloride reduction buffer containing 10 mM DTT to the extraction residue and incubate for 5 h at 37°C.
   (h) Add IAA to give a 25 mM solution, and incubate overnight in the dark at room temperature.
   (i) Pour off the supernatant corresponding to the reduced/alkylated guanidinium chloride "insoluble mucin complex".

3.1.4. Extraction of Cell-Associated Mucins from Mucus Secretions

The extracellular domains of cell-associated mucins, such as MUC1, MUC4, and MUC16, may be present in mucus secretions as a result of shedding or secretion.

1. Denaturing conditions
   To isolate them under denaturing conditions, secretions are extracted with 6 M guanidinium chloride extraction buffer in the same way as for the gel-forming species (Subheading 3.1.1, steps 1–7). Cell-associated mucins are found together with the gel-forming ones in the sol phase and/or the "guanidinium chloride soluble fraction".

2. Associative conditions
   To isolate cell-associated mucins under non-denaturing conditions, secretions should be treated as shown in Subheading 3.1.2.

3.1.5. Extraction of Cell-Associated Mucins from Mucin-Producing Cell Lines

1. Cell culture medium
   Cell-associated mucins may be extracted from aspirated cell culture medium under denaturing or non-denaturing conditions using the appropriate protocol given for extraction from secretions (Subheading 3.1.1 or 3.1.2).
2. Cell membranes
   After preparation of a membrane fraction from the cells, the extraction method used depends upon whether the whole mucin complex (transmembrane and extracellular domains) or just the extracellular domains are required. In 0.5% Triton X-100 or 0.5% sodium deoxycholate, interaction between the subunits is maintained and the whole complex can be solubilised. However, in 6 M guanidinium chloride, the transmembrane and extracellular domains dissociate and the extracellular domain is extracted leaving the transmembrane domain in the membrane (7, 8).
   (a) Wash the cells with serum-free medium, harvest using a cell scraper if necessary, and suspend in 10 mM Tris, pH 8.0. Allow to stand on ice for 2 min prior to centrifugation (600 × *g*, 2 min).
   (b) Suspend the cell pellet in ten volumes of 10 mM Tris, pH 8.0, and homogenise using four to five strokes of a Dounce homogenizer with a tight pestle.
   (c) Bring the suspension to 3 mM Mg^{2+} by adding 30 mM $MgCl_2$ and 100 mM NaCl and centrifuge (1,000 × *g*, 1 min).
   (d) Collect supernatant and centrifuge (10,000 × *g*, 10 min).
   (e) Collect supernatant and centrifuge (100,000 × *g*, 90 min). Retain pellet containing membrane fragments.
   (f) Resuspend pellet by vigorous vortexing in (1) 10 mM Tris buffer, pH 8.0, containing 0.5% Triton X-100, (2) 10 mM Tris buffer, pH 8.0, containing 0.5% sodium deoxycholate or (3) 6 M guanidinium chloride extraction buffer and incubate on ice for 30 min.
   (g) Centrifuge sample (35,000 rpm, 70.1 Ti rotor, 1 h) and retain the supernatant (see Note 11).

3.2. Mucin Purification

Although some mucus gels contain only one polymeric mucin species, e.g. MUC2 in the intestine, in general, mucus secretions contain more than one large gel-forming species (e.g. airways and cervix—MUC5AC and MUC5B; stomach—MUC5AC and MUC6). In addition, the extracellular domain of some cell-associated mucins may also be present in such secretions (e.g. airways—MUC4 and

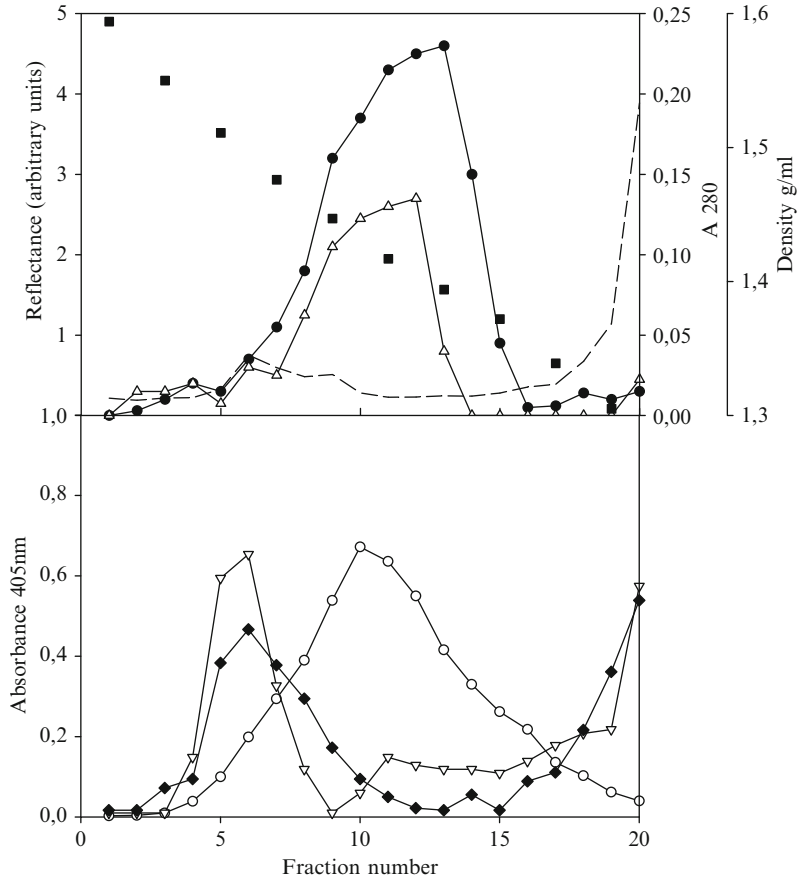


Fig. 2. Isopycnic density-gradient centrifugation in 4 M guanidinium chloride/CsCl of secretions from normal human bronchial epithelial (NHBE) cells in culture. The gel phase of apical washings from cultured NHBE cells was subjected to density-gradient centrifugation in a Beckman Optima centrifuge (36,000 rpm, 15°C, approximately 80 h, Beckman 50.2 Ti rotor) in CsCl, 4 M guanidinium chloride (starting density 1.39 g/mL). Fractions were analysed for density (*filled square*), A280 (*dashed line*), and the MAN5AC-I antibody recognizing MUC5AC (*filled circle*), LUM5B antibody recognizing MUC5B (*open triangle*), LUM16-2 antibody recognizing MUC16 (*inverted triangle*), LUM4-18 antibody recognizing MUC4 (*filled diamond*), and 214D4 antibody recognizing MUC1 (*open circle*).

MUC16). While there are some differences between the different mucins, overall they share many common structural features, in particular their high molecular weights and high sugar content. While these features allow their purification from the majority of proteins and other glycoproteins in mucus secretions, they make it difficult to separate different mucin species at the level of the intact polymer (Fig. 2). Routinely, after solubilisation using guanidinium chloride (as described above) or non-denaturing solvents such as NaCl, CsCl density-gradient centrifugation is employed to purify mucins from nucleic acids and proteins present in secretions and tissue or cell extracts. Further separation of mucins from other

smaller glycoproteins can then be achieved by gel-filtration chromatography. However, due to their similar properties (size and buoyant density), it may be difficult to cleanly separate different populations using this combined procedure. It may be possible to separate mucins by anion-exchange chromatography, but this is only achievable using mucins that have been depolymerised into their constituent monomers after treatment with reducing agents.

3.2.1. Isopycnic Density-Gradient Centrifugation Under Denaturing Conditions

This procedure can be applied to guanidinium chloride extracts of mucus secretions, tissues, cell culture medium, and cell layers (see Note 12).

1. Isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride
   (a) Dialyse samples against ten volumes of 6 M guanidinium chloride extraction buffer. The volume of the sample that can be run in each tube is two-thirds of the total volume held by the tube.
   (b) For practical purposes, the preparation of gradient is carried out by weighing rather than measuring volumes. Check the volume by weighing (the density of 6 M guanidinium chloride is 1.144 g/mL; see Note 2). If the sample volume is less than two-thirds of the total, fill up to the required volume with 6 M guanidinium chloride.
   (c) Weigh the required amount of CsCl to give the correct density into a beaker (see Note 13).
   (d) Add the sample to the CsCl stirring gently.
   (e) The final weight of the sample is calculated from the volume of the tube and the final density of the solution. Add sodium phosphate buffer to give the final weight and stir the sample gently.
   (f) Measure the density of the sample prior to loading with a syringe and cannula into the tubes. Balance the tubes carefully and seal according to the manufacturer's instructions.
   (g) Centrifuge the samples. We use a Beckman Optima LE-80 K centrifuge and a 50.2 Ti rotor (tube capacity 40 mL) with a starting density 1.39 g/mL, a 70.1 Ti rotor (tube capacity 13 mL) with a starting density of 1.40 g/mL, or a Ti 45 rotor with a starting density of 1.45 g/mL. Samples are centrifuged at 36,000 rpm (50.2 Ti rotor) or 40,000 rpm (70.1 Ti and Ti 45 rotor) at 15°C for 72–96 h. These conditions give gradients of approx. 1.25–1.60 g/mL but vary according to the rotor geometry, starting density, and speed used (see Note 11). Care should be taken to ensure that the starting concentration of CsCl at a given rotor speed and temperature does not exceed that

recommended so that CsCl does not precipitate at the bottom of the tubes during the centrifugation run. This information should be available in the manufacturer's rotor handbook.

(h) After centrifugation, recover 20–40 fractions from the gradients by piercing the bottom of the tubes and collecting fractions with a fraction collector equipped with a drop counter. Analyse the fractions for density (by weighing a known volume) and absorbance at 280 nm, as well as the appropriate carbohydrate (see Chapter 3 in this book for details) and antibody analyses (see Note 14).

(i) Large amounts of proteins/lipids in the samples, due to the use of insufficient volumes of extraction buffer, may lead to a poor separation between these molecules and mucins. In this case, mucin-containing fractions may be pooled and subjected a second time to density-gradient centrifugation in CsCl/4 M guanidinium chloride. SDS-polyacrylamide gel electrophoresis of the mucin-containing fractions may be used to determine whether all proteins have been removed.

2. Isopycnic density-gradient centrifugation in CsCl/0.5 M guanidinium chloride

Density-gradient centrifugation in CsCl/4 M guanidinium chloride may be followed by subjecting the mucin-containing fractions to a second density-gradient step in CsCl/0.5 M guanidinium chloride, which gives a better separation between mucins and DNA (see Note 15).

(a) Dialyse samples against ten volumes of 0.5 M guanidinium chloride buffer.

(b) Measure the volume of the sample by weighing (the density of 0.5 M guanidinium chloride is 1.015 g/mL; see Note 2).

(c) Weigh caesium chloride to give the required density into a beaker (see Note 13).

(d) Add the sample (volume must not exceed three-quarters of the total volume held by the tube).

(e) If required, add 1% CHAPS solution to give a final concentration of 0.01% (i.e. 1% of the total volume) (see Note 15).

(f) The concentration of guanidinium chloride in the final volume must be 0.5 M, and the volume of the CsCl and CHAPS must therefore be compensated by the addition of a small volume of 8 M guanidinium chloride.

(g) The final weight of the sample is calculated from the volume of the tube and the final density of the solution.

Add sodium phosphate buffer to give final weight and stir the sample gently.

(h) Measure the density of the sample and load into tubes with a syringe and cannula. Seal the tubes according to the manufacturer's instructions.

(i) Centrifuge the samples at 36,000 rpm (50.2 Ti rotor, starting density 1.50 g/mL) or 40,000 rpm (70.1 Ti rotor, starting density 1.52 g/mL, or Ti 45 rotor, starting density 1.52 g/mL) at 15°C for 72–96 h. These starting conditions give gradients of approximately 1.35–1.67 g/mL, but vary according to the rotor geometry, starting density, and speed used (see Note 11). Care should be taken to ensure that the starting concentration of CsCl at a given rotor speed and temperature does not exceed that recommended so that CsCl does not precipitate at the bottom of the tubes during the centrifugation run. This information is available in the manufacturer's rotor handbook.

(j) After centrifugation, recover 24–40 fractions from the gradients by piercing a hole in the bottom of the tubes and collecting fractions with a fraction collector equipped with a drop counter or by manually counting drops. Analyse the fractions for density (by weighing a known volume) and absorbance at 280 nm, as well as the appropriate carbohydrate (see Chapter 3 in this book for details) and antibody analyses (see Note 14).

3.2.2. Isopycnic Density-Gradient Centrifugation Under Associative Conditions

This procedure can be applied to extracts of mucus secretions, cell culture medium, and membranes from cultured cells obtained without the use of guanidinium chloride (see Note 16).

Isopycnic Density-Gradient Centrifugation in CsCl/0.1 M Sodium Chloride

1. Check the volume of the sample by weighing (it should not exceed 4/5 of the total volume held by the tube).
2. Weigh the required amount of CsCl to give the correct starting density into a beaker (see Note 13).
3. Add the sample to the CsCl stirring gently.
4. The final weight of the sample is calculated from the volume of the tube and the final density of the solution. Add 0.1 M sodium chloride if necessary to give the final weight and stir gently.
5. Measure the density of the sample prior to loading with a syringe and cannula into the tubes. Balance the tubes carefully and seal according to the manufacturer's instructions.
6. Centrifuge the samples. We use a Beckman Optima LE-80 K centrifuge and a 50.2Ti rotor (tube capacity 40 mL) with a starting density of 1.45 g/mL, a 70.1 Ti rotor (tube capacity 13 mL)

with a starting density of 1.40 g/mL, or a Ti 45 rotor with a starting density of 1.45 g/mL. Samples are centrifuged at 36,000 rpm (50.2Ti rotor) or 40,000 rpm (70.1 Ti and Ti 45 rotor) at 15°C for 72–96 h. These conditions give gradients of approx 1.30–1.60 g/mL but vary according to the rotor geometry, starting density, and speed used (see Note 11). Care should be taken to ensure that the starting concentration of CsCl at a given rotor speed and temperature does not exceed that recommended so that CsCl does not precipitate at the bottom of the tubes during the centrifugation run. This information should be available in the manufacturer's rotor handbook.

7. After centrifugation, recover 20–40 fractions from the gradients by piercing the bottom of the tubes and collecting fractions, either using a fraction collector equipped with a drop counter or by measuring volume. Analyse the fractions for density (by weighing a known volume) and absorbance at 280 nm, as well as the appropriate carbohydrate and antibody analyses (see Note 14).

3.2.3. Gel Chromatography

1. Sephacryl S-1000, Sepharose CL-2B, Sepahacryl S-500, or Superose 6
   (a) Elute columns with 4 M guanidinium chloride buffer at flow rates well below the maximum recommended for each column.
   (b) Dialyse samples against 4 M guanidinium chloride and apply them to the column through a loading loop.
   (c) Monitor the eluate online with an ultraviolet (UV) monitor and collect fractions using a fraction collector and subject them to the appropriate carbohydrate (see Chapter 3 in this book for details) and antibody analyses (see Note 14).

3.2.4. Ion-Exchange Chromatography

Run Mono Q or Resource Q columns on a system comprising an HPLC system with an automated injector. We routinely use an Akta Purifier at a flow rate of 0.5 mL/min. All connections are made using Teflon or Peak tubing.

1. Equilibrate the column with ten volumes of buffer A or buffer C.
2. Dialyse the sample exhaustively or dissolve sample in buffer A or buffer C and apply the sample to the column.
3. Run the column with 5 volumes of buffer A or buffer C and then with a linear gradient up to 100% buffer B or buffer D (gradient equivalent to 30-column volumes).
4. Monitor the eluate online with a UV monitor and collect fractions (we collect 0.5 mL) using a fraction collector and subject to the appropriate carbohydrate (see Chapter 3 in this book for details) and antibody analyses (see Note 14).

3.2.5. Agarose Gel Electrophoresis and Transfer to Membrane Support

1. Dissolve agarose in running buffer in a microwave. We routinely use 0.7% gels for intact mucin polymers and 0.7–1.0% gels for reduced mucins.
2. Allow to cool to around 40°C and pour the gel into the casting trough. Insert well-forming comb (see Note 17).
3. Leave to set for at least 1 h prior to use.
4. Load sample in situ in the gel tank and then submerge gel with running buffer. Run samples for 16 h at 30 V at room temperature. Alternatively, electrophoresis can be performed for 3 h at 30 V.
5. Transfer the gel to transfer buffer for 5 min. If samples were unreduced, the mucins can be reduced by incubating the gel with 10 mM DTT in transfer buffer for 30 min at room temperature prior to transfer (see Note 18).
6. Transfer mucins to membrane support (nitrocellulose or PVDF) by vacuum blotting (we use a Vacu-Gene XL); suction pressure 45 mBar for 2 h.
7. The membrane can be stained with PAS reagent (see Chapter 3 in this book for details) or probed with mucin-specific antisera (see Note 14).

4. Notes

1. While commercially available preparations such as freeze-dried porcine gastric and bovine submaxillary mucins can be used for some purposes, they contain mixtures of gel-forming mucins, which, in our experience, are often highly degraded. They are, thus, of limited value for use in most of the applications presented here and this should be taken into account when interpreting data obtained using such preparations.
2. The molarity of a guanidinium chloride solution can be calculated from the density according to the following formula:

$$M = \frac{\rho - 1.003}{0.02359},$$

where M is the molarity and ρ is the density in grams per millilitre.

3. Inhibitors are added to the 6 M guanidinium chloride extraction buffer in order to block the activity of the three classes of proteolytic enzymes: metalloproteases, serine proteases, and thiol proteases. The action of metalloproteases is inhibited by the addition of 5 mM sodium EDTA to the buffer. This can be added during the initial preparation of the buffer since it is

stable at room temperature. Thiol proteases are inactivated by the addition of 5 mM NEM. NEM has the added advantage that it blocks exchange reactions between free thiol groups and disulphide bonds. DFP is a potent inhibitor of serine proteases and esterases, including acetylcholinesterase, and should therefore be handled with extreme care. DFP is supplied in 1-g vials with a septum, and prior to dilution vials should be cooled on ice to reduce the vapour pressure. Working in a fume cupboard, the septum should be pierced with a needle to equilibrate the pressure, and the DFP should be transferred using a syringe and needle. The contents of the vial should be placed directly into the correct volume of ice-cold dry propan-1-ol to give a 100 mM solution. DFP is unstable in water but can be stored at −20°C in propan-1-ol. After dilution, the vial as well as the needles and syringes should be rinsed with 1 M NaOH to inactivate the DFP. PMSF can be used at a concentration of 0.1 mM in place of DFP. However, the low solubility of PMSF in water makes it a less attractive option, although it is possible to prepare a stock solution of PMSF in an organic solvent which is miscible with water.

4. Reduced subunits (mucin monomers) can be prepared from polymeric gel-forming mucins by treatment with reducing agents. For this purpose, the sample should be dialysed into, or dissolved in, 6 M guanidinium chloride reduction buffer. To break the disulphide bonds between the monomers, the reducing agent DTT should then be added to give a final concentration of 10 mM and the sample incubated for 5 h at 37°C. IAA is then used to alkylate the free thiol groups and prevent their re-association. IAA should be added to give a 25 mM solution and the sample maintained overnight at room temperature in the dark.
5. In our laboratories, we generally use CsCl, rather than CsBr or Cs_2SO_4, as the density-gradient forming salt since our gradients are often run in the presence of guanidinium chloride and the use of CsBr or $CsSO_4$ would give rise to mixed caesium salts. Gel-forming MUC5B mucins and nucleic acids have, however, been shown to be well-resolved in density gradients of Cs_2SO_4/10 mM sodium phosphate buffer, pH 6.5, whereas the separation obtained in CsBr/10 mM sodium phosphate buffer, pH 6.5, is poor (9).
6. In our experience, mucins show a tendency to adhere to Sephacryl columns, which is seen to a lesser extent with the Sepharose CL-2B or Superose 6 gels.
7. Sephacryl S-500HR and Superose 6 columns can be purchased pre-packed or packed in the laboratory into custom-sized columns according to manufacturer's instructions. Sepharose

CL-2B and Sephacryl S-1000 columns cannot be purchased pre-packed and must be prepared in the laboratory. The columns should be sufficiently large so that sample volumes do not exceed 5% of the total column volume. Sephacryl S-1000 gel is sufficiently porous for some polymeric gel-forming mucin species, e.g. MUC5B from saliva, to be separated from each other. On Sepharose CL-2B and Sephacryl S-500 columns, the whole polymeric gel-forming mucins, as well as the cell-associated MUC16 mucin, elute in the void volume and can thus be resolved from reduced subunits or cell-associated species, such as MUC1, which are included on the gels. Superose 6 columns are of value for the separation of reduced mucin subunits that elute in the void volume from mucin glycopeptides which elute in a more included volume.

8. We use lithium perchlorate as the eluting salt because many of the assays we originally employed were compatible with this salt; however, sodium chloride could be used instead. The technique we employ works well for cell-associated mucins and reduced mucin subunits from the gel-forming species but not for intact polymeric mucins. For the reduced subunits, 6 M urea and CHAPS are included in the buffer (buffers C and D) to minimise non-specific hydrophobic interactions with the column matrix that can occur as a result of opening up hydrophobic regions of the polypeptide by reduction.
9. For mucus gels from most sources, the degree to which the large polymeric mucins are solubilised by treatment with guanidinium chloride depends largely on the extraction conditions. Our operational definition of the "guanidinium chloride insoluble" fraction is the material that appears in the centrifugation pellet after three rounds of extraction with 6 M guanidinium chloride extraction buffer. However, for MUC2 mucus gels from the intestine, most of the material is resistant to extraction with 6 M guanidinium chloride extraction buffer and is only brought into solution after reduction and alkylation. The presence of an additional cross-linking bond that stabilises the MUC2 gel network has been postulated to account for this observation (10).
10. Preparation of mucins from mucus secretions without the addition of guanidinium chloride as a solubilising agent can lead to enrichment of non-gel-forming species or degraded molecules since complexes of gel-forming mucins may be pelleted during the centrifugation step.
11. The rotor type, speed, and starting densities rather than the g-force are given for the density-gradient runs. In our experience, for fixed angle rotors such as the 70.1Ti, 50.2Ti, or Ti45, the rotor geometry (the tube angle within the rotor), starting

density, and speed at which the run is conducted are the most important factors determining the gradient formed and the conditions cannot necessarily be reproduced using the same g-force in another rotor.

12. For cell-associated mucin complexes (e.g. MUC4α and MUC4β or ASGP-1 and ASGP-2), isolated by detergent extraction from cell membranes, isopycnic density-gradient centrifugation in the presence of guanidinium chloride leads to dissociation of the two subunits in the gradient.
13. The amount of caesium chloride needed to give a required density in 4 M or 0.5 M guanidinium chloride can be calculated according to the following formula:

$$x = v\,(1.347\rho - 0.0318M - 1.347),$$

where x is CsCl (grams), v is the final volume, M is the molarity of the guanidinium chloride (4 or 0.5), and ρ is the starting density (grams per milliliter). If no guanidinium chloride is used, the amount of caesium chloride needed (x) is equal to the final volume (v) multiplied by the starting density (ρ).

14. A range of different antibodies is available for the detection of gel-forming and cell-associated mucins. Table 1 shows antibodies that have been used successfully in our laboratories for this purpose.
15. Some mucins show a tendency to precipitate in the presence of CsCl at low concentrations of guanidinium chloride, and 0.1% CHAPS is sometimes added to the gradient to counteract this effect.
16. For complexes of cell-associated mucins isolated from cell membranes using Triton X-100 extraction, Triton X-100 or sodium deoxycholate should be added to the gradient to give a final concentration of 0.5% in the solution. Under associative conditions (i.e. in the absence of guanidinium chloride), no dissociation between the subunits should occur in the gradient.
17. The comb size (width relative to thickness) is an important factor in the quality of the data. Thin combs yield better quality data resulting in sharper bands.
18. Because of the large size of the intact polymers, their transfer from the gel onto the membrane support (needed for mucin detection) is problematic. Therefore, prior to vacuum transfer, the mucin polymers are converted into their constituent monomers.

Table 1
Antibodies used for the detection of mucin proteins

Mucin	Antibody name	Recognition site	Supplier/references
MUC1	214D4 (mouse monoclonal)	Extracellular domain	Millipore
MUC2	LUM2-3 (rabbit polyclonal)	C-terminal side of large tandem repeat	(11)
MUC4	LUM4-18 (rabbit polyclonal)	Extracellular domain	Not published
MUC5AC	LUM5-1 (rabbit polyclonal)	Unique sequences flanking tandem repeats	(11, 12)
	MAN5AC-I (rabbit polyclonal)	Unique sequences flanking tandem repeats	(13)
	45 M1 (mouse monoclonal)	Unique sequences flanking tandem repeats	Abcam, Santa Cruz
	Mab2011	Tandem repeat (precursor)	Chemicon Int. CA
MUC5B	LUM5B-2 (rabbit polyclonal)	Cysteine-rich domains of central region	(14)
	LUM5B-3 (rabbit polyclonal)	Cysteine-rich domains of central region	(14)
	LUM5B-4 (rabbit polyclonal)	C-terminal domain	(14)
	MAN5B-I (rabbit polyclonal)	Cysteine-rich domains of central region	(15)
	MAN5B-III (rabbit polyclonal)	Unique sequence flanking tandem repeats	(16)
	EUMUC5B-2a (mouse monoclonal)	Cysteine-rich domains of central region	(17)
	EUMUC5B-2b (mouse monoclonal)	Cysteine-rich domains of central region	(17)
	LUM5B-13 (rabbit polyclonal)	Tandem repeat (precursor)	Not published
MUC6	LUM6-1 (rabbit polyclonal)	Tandem repeat	(18)
MUC7	LUM7-1 (rabbit polyclonal)	N-terminal of tandem repeat domain	(19)
	LUM7-2 (rabbit polyclonal)	N-terminal of tandem repeat domain	(19)
	EUMUC7-1a (mouse monoclonal)	N-terminal of tandem repeat domain	(17)
MUC16	LUM16-2 (rabbit polyclonal)	Extracellular domain	(20)
	OC125 (mouse monoclonal)	Extracellular domain	NeoMarkers, CA, USA
	M11 (mouse monoclonal)	Extracellular domain	NeoMarkers, CA, USA
	LUM16-4 (rabbit polyclonal)	Cell-associated domain	(20)

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