

Chapter 2

Materials and Methods

This chapter contains the theory and protocols of experimental techniques used in this work, as well as details on the materials and methods for each experiment that was performed. First, general materials and methods are summarised, followed by a description of more specialised methods such as chromatography techniques and nanoparticle characterisation methods. The protocol for MF synthesis and cationisation is included, as well as basic protocols for stem cell culture. After that, experiments carried out to investigate stem cell labelling with cat-MF are described, followed by the methods that were used to assess cat-MF toxicity. Finally, bacterial culture protocols are described, as well as the experiments conducted to investigate cat-MF labelling of bacteria.

2.1 General Materials

Unless otherwise stated, chemicals were purchased from Sigma Aldrich (UK) and used as received. Deionised water (dH_2O) was obtained from a Merck Millipore ultrapure water system, with a resistivity of approximately $18 \text{ M}\Omega \text{ cm}$.

2.2 General Methods

2.2.1 Buffer Preparation

Theory

Buffer solutions prevent a rapid change in pH when acids or bases are added, such that the pH of a solution can be maintained at a near constant value. This is important in, for example, biochemical applications because many proteins and

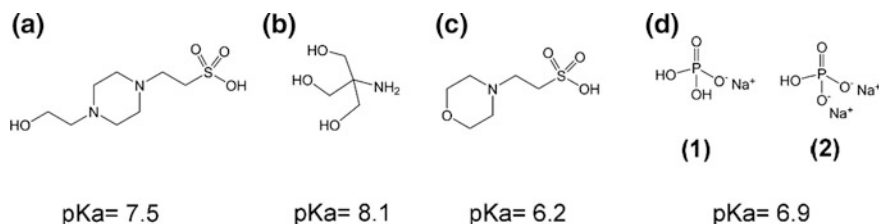


Fig. 2.1 Chemical formulae of buffering agents and their pK_a values at 25 °C. **a** HEPES, **b** Tris, **c** MES, and **d** phosphate buffer components: monosodium phosphate (1) and its conjugated base disodium phosphate (2)

enzymes only retain their structure and function over a narrow pH range [1]. There are a variety of buffering agents, such as Tris, HEPES and MES that have different buffering ranges. Mixtures of acids and their conjugate base can also be used to make a buffer solutions, as is the case with phosphate buffers (Fig. 2.1).

Buffer solutions resist pH change because of the equilibrium between the acid (HA) and conjugate base (A⁻) form of the buffering agent or the acid-base pair.



When acid is added, the additional protons are “captured” by the conjugate base form of the buffer agent and the equilibrium shifts towards the left hand side. The opposite is true when base is added. At a certain pH value, the concentration of acid and conjugate base is equal. This is described by the pK_a value. Thus, the pH changes relatively slowly in the buffer region of pH = pK_a ± 1.

Method

Buffer salts were dissolved in 80–90% of the final volume of dH₂O, the pH was adjusted with dilute hydrochloric acid (HCl) or sodium hydroxide solutions (NaOH) to the desired value, and the buffer solution made up to the final volume with dH₂O. Stock solutions were prepared for all buffers, which were diluted to the desired concentrations as required for individual experiments.

HEPES buffer was prepared as a 500 mM stock solution using 119 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in one litre dH₂O and the pH adjusted to 8.6 with NaOH.

Tris buffer was prepared as a 500 mM stock solution by dissolving 60.5 g of tris (hydroxymethyl)aminomethane (Tris) in one litre dH₂O, and the pH adjusted to pH 8.0, 7.6, or 7.0 using HCl.

Phosphate buffer (pH 7) was prepared from 57.2 g monosodium phosphate and 74.2 g disodium phosphate in one litre dH₂O to yield a 1 M stock solution.

MES buffer (pH 5) was prepared as a 200 mM stock by dissolving 39 g of 2-(N-morpholino)ethanesulfonic acid (MES) in one litre dH₂O and adjusting the pH with NaOH.

2.2.2 UV/Visible Spectroscopy

Theory

UV/visible spectroscopy is commonly performed on molecules in solution, and can be used to determine electronic transitions in those molecules, [2] or to assess scattering intensity from a suspension of bacteria [3]. Irradiation with a certain wavelength (λ) of the UV/visible spectrum can lead to strong absorption of the light's energy and subsequent excitation of the molecule's valence electrons to higher energy states. The molecule is said to have an absorption maximum at this wavelength. If the absorption maximum is unknown, samples can be scanned with a range of wavelengths. Plotting the measured absorbance against wavelength reveals the characteristic absorbance peaks for the molecule(s) present in the solution. The level of absorption at the absorption maximum is proportional to the molecule's concentration, and can be used for quantification [4].

Absorption measurements are also employed to determine the turbidity in a bacterial suspension, which gives an indication of cell density and therefore bacterial number. These measurements are actually based on the amount of light scattered from the bacteria, rather than absorption of light. To maximise light scattering, UV/visible spectroscopy is performed using wavelengths that are not absorbed by the molecules in the bacterial cell.

Absorption is measured using a spectrophotometer. In general, this machine consists of two devices: a spectrometer that can produce a desired range of wavelengths of light, and a photometer that detects the amount of photons transmitted through the sample. The amount of transmitted light depends on the path length through the sample and the concentration of molecules in solution. Thus, the path length needs to be kept constant if the concentration is to be determined. The transmittance, T , is the fraction of light that passes through the sample and can be calculated as follows:

$$T = \frac{I_t}{I_0} \quad (2.1)$$

where I_t is the intensity of the transmitted light and I_0 is the intensity of the light before it passed through the cuvette. Transmittance is related to absorption, A , by the expression:

$$A = -\log(T) \quad (2.2)$$

The more light is transmitted, the lower the absorbance and vice versa. For example, if 80% of the light is transmitted through the sample, the absorption value will be approximately 0.1, whereas if only 50% of the light is transmitted, the absorption will be 0.3 (Fig. 2.2a). In the case of measuring the turbidity of a sample to give an indication of cell number, light scattering results in fewer photons reaching the detector, i.e. a loss in transmittance and therefore an increased absorbance value (Fig. 2.2b).

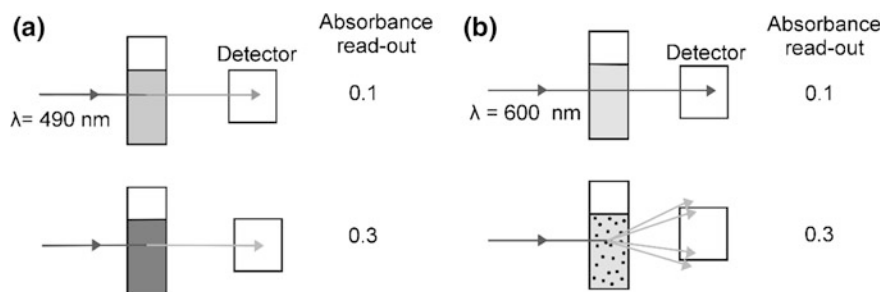


Fig. 2.2 UV/Visible spectroscopy setups. **a** Measuring absorbance using a wavelength at which the molecule has an absorption maximum. The level of absorption is proportional to the molecule's concentration. **b** Measuring the absorption in a bacterial suspension with a wavelength that is not absorbed by any components of the bacterial cell. The degree of scattering is reflected in the absorbance read-out

Method

UV/visible spectroscopy of bacterial cultures was performed in an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, UK) using $\lambda = 600$ nm. For the measurements, 0.7 mL of background solution (sterile dH₂O or culture medium) or bacterial solution were pipetted into disposable semi-micro cuvettes (VWR, UK) and placed in the spectrophotometer. Absorbance of the background solution was subtracted from the absorbance of the bacterial culture.

All other UV/visible spectroscopy measurements were performed in microtitre plates to allow high-throughput measurement of large numbers of samples. The samples were placed in individual wells and irradiated sequentially in a SpectraMax M5e spectrophotometer (Molecular Devices, UK) using an appropriate wavelength. Background solutions were included in the measurement and subtracted from the samples' absorbance values.

2.2.3 Fluorescence Spectroscopy

Theory

Fluorescence spectroscopy is used to measure light emission from fluorophores in solution. Orbital electrons in the fluorophore are excited into high energy electronic states by the absorption of incident photons. When the electrons relax back to the ground state they lose energy through emission of a photon of longer wavelength (lower energy) than the absorbed one (Fig. 2.3a). This so-called Stokes shift is due to non-radiative relaxation, meaning that the electrons lose some of their energy through molecular vibrations, before they return to the ground state [5]. In fluorescence spectroscopy, the sample is irradiated with light of a specific wavelength (excitation). Emitted photons are collected with a detector placed perpendicular to the excitation beam (Fig. 2.3b).

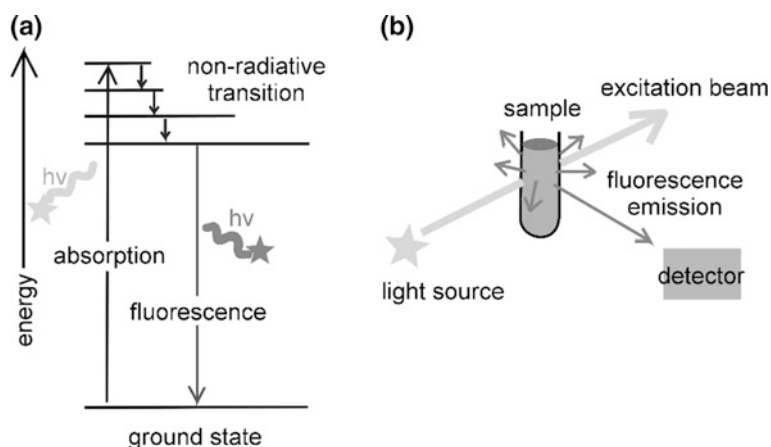


Fig. 2.3 Fluorescence spectroscopy. **a** Photons of a certain wavelength can excite the orbital electrons from a fluorophore to higher energy states. The excited electrons can lose some of their energy due to molecular vibrations, such that when they relax back to the ground state, they emit photons with a longer wavelength (less energy) than the absorbed photons. **b** Basic setup to measure fluorescence. The sample is irradiated with light of a specific wavelength (excitation), and emitted photons are collected with a detector placed perpendicular to the excitation beam

Method

Fluorescence spectroscopy measurements were performed in microtitre plates to allow high-throughput measurement of large numbers of samples. The samples were placed in individual wells and irradiated sequentially in a SpectraMax M5e spectrophotometer (Molecular Devices, UK) using an adequate excitation and emission wavelength.

2.2.4 Bradford Assay

Theory

The Bradford assay is a colorimetric method to determine protein concentration using the dye Coomassie Brilliant Blue G250 [6]. The dye exists in three forms, which have distinct colours: cationic (red), neutral (green) and anionic (blue). Under acidic conditions, the dye is predominantly in the doubly protonated (cationic) form, with an absorbance maximum at $\lambda = 470$ nm. When the dye is added to a protein solution it binds to basic amino acids (mainly arginine residues) and is converted to the de-protonated (anionic) form with an absorbance maximum at $\lambda = 595$ nm (Fig. 2.4). By measuring the absorbance at $\lambda = 595$ nm of a series of protein standards, the protein concentration of an unknown sample can be determined.

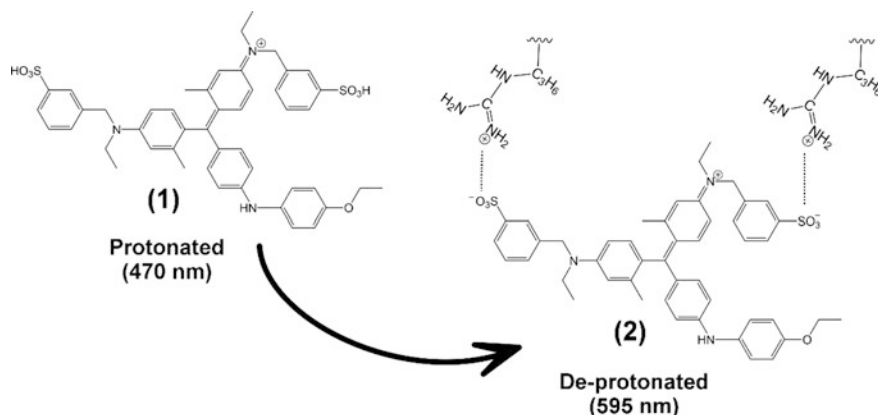


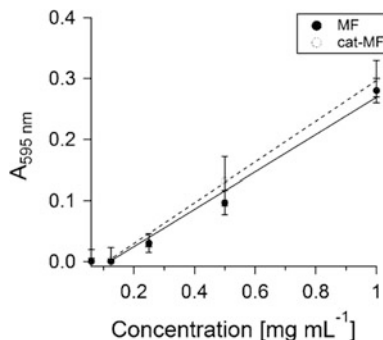
Fig. 2.4 Chemistry underlying the Bradford assay. The protonated form of the dye Coomassie Brilliant Blue G250 (1) predominates under acidic conditions. Binding of arginine residues on proteins causes a shift in absorbance maximum from 470 nm to 595 nm (2)

Method

Ferritin standards were prepared from horse spleen ferritin solution, for which the protein concentration had been determined by the supplier. Standards of 0.06, 0.125, 0.25, 0.5 and 1 mg mL⁻¹ were prepared in phosphate buffer (50 mM, pH 7). Unknown samples of MF or cat-MF were diluted in phosphate buffer (50 mM, pH 7) until their colour approximately matched the colour of the 0.5 mg mL⁻¹ standard. This was done to ensure the sample absorption would lie within the absorption values of the standard curve. 10 µL of each standard and unknown sample was pipetted out in triplicate into wells of a 96 well plate, 200 µL of Bradford reagent was added to each well and the plate was incubated at room temperature for 8 min. Absorbance at $\lambda = 595$ nm was measured using UV/visible spectroscopy and used to calculate the protein concentration of the unknown MF or cat-MF samples. The mean absorbance of the ferritin standards was plotted as a function of protein concentration and the slope and intercept of the linear fit were used to calculate the concentration of the unknown protein sample.

To evaluate whether cationised residues on the cat-MF surface interfered with the Bradford assay, 0.06, 0.125, 0.25, 0.5 and 1 mg mL⁻¹ of MF and cat-MF were prepared in phosphate buffer (50 mM, pH 7), and the Bradford assay was carried out as described above. Absorbance values and slopes of the linear fit were similar and variations of absorbance values between MF and cat-MF were within error (Fig. 2.5). Therefore, it was concluded that cationisation did not affect the accuracy of the Bradford assay.

Fig. 2.5 Bradford assay carried out on MF and cat-MF samples ($n = 2$). The slopes of the linear fit are 0.31 for MF (*solid line*), and 0.33 for cat-MF (*dashed line*). Absorbance values are similar for MF and cat-MF



2.3 Chromatography Methods

Chromatography is a technique for the separation of a mixture of components in a solution or suspension. The mixture is passed through a medium in which the components interact more or less strongly with the medium depending on particular properties, such as charge or size. Therefore, they move through the medium at different rates and can be collected in sequential fractions. Here, two chromatography techniques were used to purify MF after synthesis. During MF synthesis, nanoparticles form inside the ApoF cavity, as well as in the buffer solution. Thus, ion exchange chromatography was employed to separate MF from the free nanoparticles. Agglomerates of MF were then separated from MF monomers using size exclusion chromatography. All chromatography methods were performed using an ÄKTApurifier system (GE Healthcare, UK).

2.3.1 Ion Exchange Chromatography

Theory

Ion exchange chromatography (IEC) separates proteins based on their charge [7]. Reversible interactions between charged molecules and the oppositely charged ion exchange matrix are controlled to achieve binding or elution of particular molecules, resulting in their separation. The ion exchange matrix consists of porous, spherical particles substituted with ionic groups that are negatively or positively charged. The pH plays an important role in IEC of proteins, because proteins have no net charge at a pH equivalent to their isoelectric point and therefore won't interact with a charged medium. However, at a pH above their isoelectric point, proteins will bind to a positively charged matrix and at a pH below it proteins bind to a negatively charged matrix. Both pH and ionic strength of the buffer are selected such that proteins of interest bind to the matrix when the sample is loaded, while unwanted material does not bind and can be washed out with buffer (Fig. 2.6a, b). To elute the bound protein the buffer conditions are changed, for example by

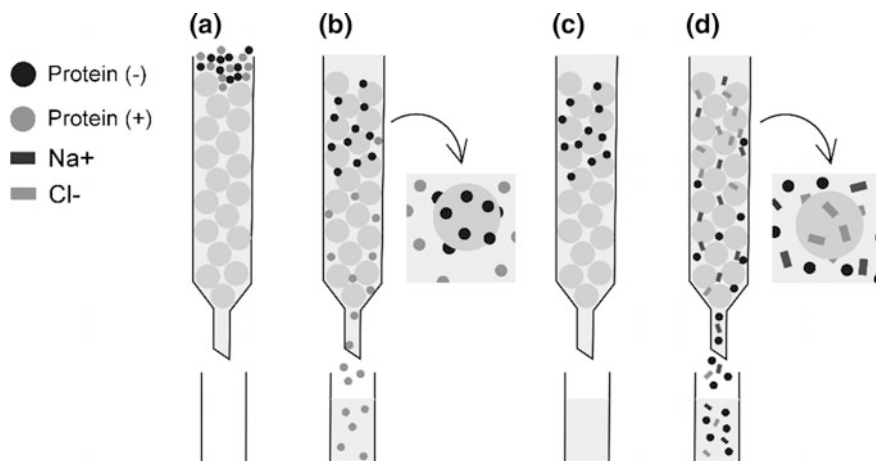


Fig. 2.6 Schematic of ion exchange chromatography. **a** A mixture of proteins with different charges at a given pH is loaded onto the column containing a charged matrix. **b** Proteins with a charge opposite to that of the matrix are immobilised, while all other components are eluted. **c** A wash step is included to remove any material that is not immobilised, such that only the target protein remains trapped in the column. **d** Elution of the target protein is achieved by increasing the ionic strength of the buffer. As ionic strength increases, the salt ions compete with the bound proteins for charges on the matrix surface. Bound molecules are detached, move down the column and can be collected

increasing the ionic strength (salt concentration) of the buffer. As ionic strength increases, the salt ions (typically Na⁺ or Cl⁻) compete with the bound proteins for charges on the matrix surface such that bound molecules detach and move down the column (Fig. 2.6d). The components with the lowest net charge at the selected pH will elute first from the column, while higher charged species will be more strongly bound and elute as ionic strength increases. Results of IEC are displayed as an elution profile generated by a UV/visible spectroscopy cell incorporated into the purifier setup. The absorbance of the solution is monitored at a particular wavelength (e.g. 280 nm) as it exits the column. Peaks in the absorbance indicate when proteins are eluted.

Method

Samples were loaded onto a column (2.5 cm diameter, 20 cm long) containing the cationic matrix ANX Sepharose 4 Fast Flow (GE Healthcare, Life Sciences) using a P960 sample pump (GE Healthcare, UK) at a flow rate of 10 mL min⁻¹. Tris buffer (50 mM, pH 8) was used as a running buffer (flow rate 10 mL min⁻¹), with which metal oxide nanoparticles were eluted from the column, while protein nanoparticles were immobilised on the matrix. To elute the protein, Tris buffer containing increasing concentration of NaCl was added (from 100 to 1000 mM) at the same flow rate. The protein eluted at a NaCl concentration of 500 mM and was collected in 50 mL fractions using an automated fraction collector (Frac-950, GE Healthcare, Life Sciences).

2.3.2 Size Exclusion Chromatography (SEC)

Theory

Size exclusion chromatography (SEC) separates the components in a sample based on differences in size as they pass through a filtration medium packed inside a column [8]. The filtration medium is a porous matrix that consists of spherical particles (Fig. 2.7a). The matrix is equilibrated with buffer, which fills all pores and spaces. Thus, it forms a stationary phase that is in equilibrium with the buffer that is pumped through the column (mobile phase). Unlike IEC, molecules passed through the column in the mobile phase do not bind to the filtration medium, but are eluted from the column in order of their molecular size. Larger molecules that cannot enter the pores of the matrix are eluted first, while smaller molecules with partial access to the pores elute in order of decreasing size (Fig. 2.7b, c). Very small molecules, such as salts, have full access to the pores and move down the column very slowly. Results from SEC are displayed as an elution profile (see Sect. 2.3.1).

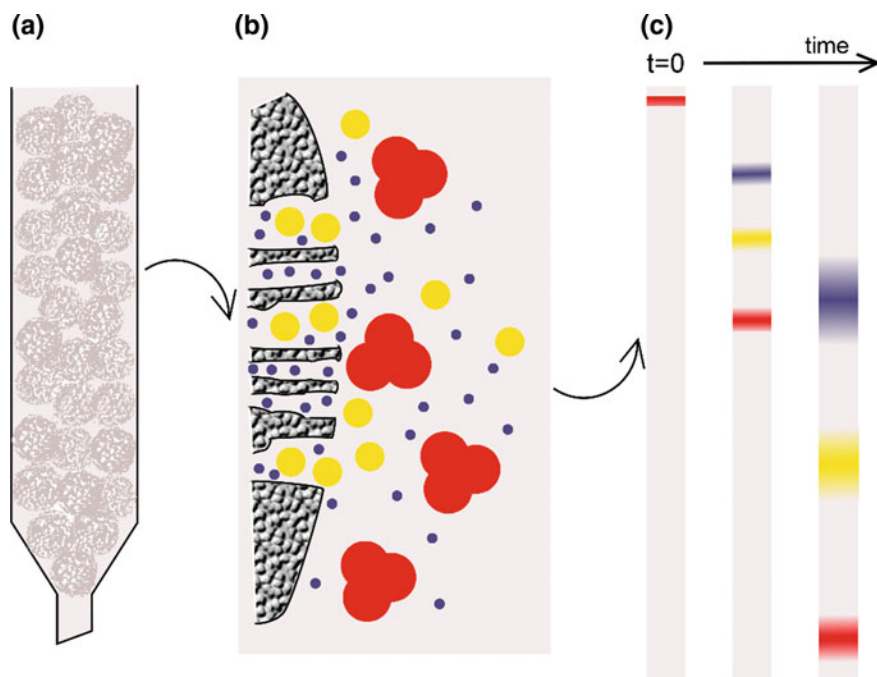


Fig. 2.7 Size exclusion chromatography. **a** A column is packed with a matrix of spherical, highly porous particles. **b** When the sample is passed through the column matrix, components in the sample can access pores in the matrix according to their size. **c** Schematic of the gel filtration process over time. The sample is loaded onto the column at $t = 0$. Over time, the components of the sample pass through the length of the column and are separated according to their sizes (*blue* smallest molecules, *red* largest molecules, *yellow* middle-sized molecules)

Method

MF samples pre-purified using IEC were concentrated using Centriprep centrifugal filter units (Merck Millipore, UK) to a volume of approximately 2 mL. They were loaded onto a HiPrep 26/60 Sephacryl S-300 h column (GE Healthcare, Life Sciences) column using an injection loop. Tris buffer (50 mM, pH 8.0) containing 150 mM NaCl was used as running and elution buffer at a flow rate of 1.6 mL min^{-1} . Fractions of 6 mL volume were collected using an automated fraction collector (Frac-950, GE Healthcare, Life Sciences).

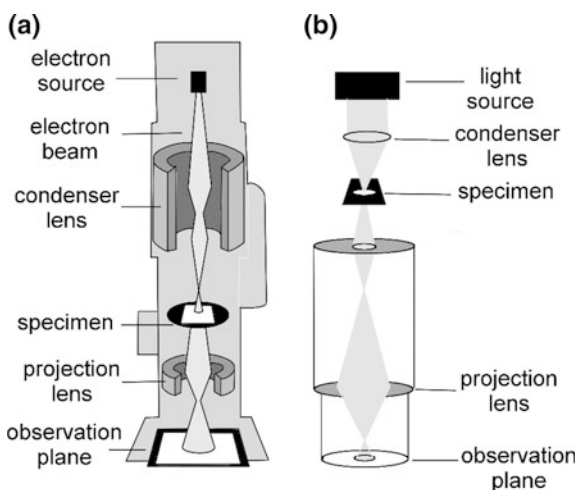
2.4 Characterisation Methods

2.4.1 Transmission Electron Microscopy

Theory

The working principle of TEM is similar to that of light microscopy in that the image of a specimen is magnified and focussed onto an observation plane (Fig. 2.8a, b). The crucial difference is the type of energy beam used to “illuminate” the specimen: while light microscopy employs visible light beams of wavelengths between 400 and 700 nanometres to observe a sample, an electron beam of wavelengths in the low picometre range is used for electron microscopy. Therefore, it is possible to observe very fine sample features using TEM. Features about 0.2 nm apart can be resolved with TEM, [9] whereas maximum resolution in light microscopy is about 200 nm [10]. Consequently, TEM is frequently used to characterise shape and size of nanomaterials, but also their crystal phase and composition can be determined.

Fig. 2.8 Schematic of microscopic techniques. **a** In a transmission electron microscope (TEM) an electron beam is generated, which is focussed through electromagnetic lenses onto the specimen and eventually onto an observation plane on a fluorescent screen or a digital camera. **b** Analogous, in a light microscope a visible light beam is focussed through glass lenses onto a specimen and then onto an observation plane



The most common mode of operation in TEM is the bright field imaging mode. In this mode, the contrast of the TEM image is due to absorption and scattering of electrons in the sample. Thick samples or materials with a high atomic number scatter electrons more strongly and will appear dark, while regions without sample in the beam path appear bright (hence the name “bright field”). However, the elements that make up organic materials, such as proteins and lipids, have very low atomic numbers and are therefore “invisible” in a TEM image. To visualise these types of materials, a variety of metal-based stains are employed. These can be negative stains, which surround organic materials such that they appear as bright structures on a dark background. For example, the protein capsules of viruses are commonly negative stained with neutralised phosphotungstic acid [11]. Stains can also bind to specific biological structures like phospholipid membranes, proteins and nucleic acids. To visualise these structures in cell sections, the cells should first be fixed to avoid structural changes occurring during the staining procedure. Common fixatives are glutaraldehyde, which cross-links proteins in the cell through methylene bridges, [12] as well as osmium tetroxide, which mainly reacts with the carbon-carbon double bond in unsaturated fatty acids chains in cell membranes [13]. Uranyl acetate is routinely used for contrast staining of biological structures because uranyl ions bind to carboxyl groups of proteins and lipids, and to phosphate groups of nucleic acids. Therefore, uranyl acetate staining achieves good contrast staining of membranes, nucleic acids and protein complexes associated with nuclei acids, such as ribosomes [14]. Staining with lead citrate enhances the contrast for several cellular structures such as phospholipid membranes, ribosomes and the cytoskeleton, particularly after osmium tetroxide fixation [15].

Methods

A variety of preparation methods was used in this work depending on the sample to be imaged.

To confirm **nanoparticle mineralisation** and determine MF core size and overall diameter, 10 μL samples containing approximately 0.5 mg mL^{-1} protein were placed onto carbon-coated copper grids (Agar Scientific, UK) for 10 min. The excess was removed with filter paper and either left to dry or stained with 2% (w/v) neutralised phosphotungstic acid (pH 7) for 30 s before air drying. Samples were observed using an acceleration voltage of 120 kV on a JEM 1200 EX transmission microscope (JEOL, UK) and images recorded with a MegaView II digital camera (Olympus, UK) running analySIS 3.0 software (Soft Imaging Systems GmbH, Germany).

To observe **stem cells** exposed to cat-MF, cells were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and with 0.5% (w/v) osmium tetroxide in cacodylate buffer, each for 30 min at room temperature. Fixed cells were washed in cacodylate buffer and distilled water, before dehydration in a graded series of ethanol (from 70% to 100%). After that, they were embedded in an epoxy resin (Epon), and the resin polymerised at 60 °C for 24 h. Thin sections of 70 nm thickness were cut from the resin blocks and transferred onto carbon-coated copper grids. Sections were contrast stained with 3% (w/v) uranyl acetate and 0.5% (w/v) alkaline lead citrate. Localisation of cat-MF on labelled cells was investigated

using a Tecnai 12 BioTwin Spirit transmission electron microscope (FEI, UK) with an acceleration voltage of 120 kV. Images were recorded with an Eagle 4 k camera (FEI) running TEM Imaging and Analysis software (FEI).

To image **bacteria** exposed to cat-MF, 10 μL samples of bacterial suspension were placed onto carbon-coated copper grids for 10 min. The excess was removed with filter paper and grids were floated on 100 μL drops of 5% (v/v) glutaraldehyde solution for 30 min at room temperature to fix the cells. After that, grids were washed by placing them on three 100 μL drops of dH_2O for 5 min each and air dried. Samples were observed using an acceleration voltage of 120 kV on a JEM 1200 EX transmission microscope and images recorded with a MegaView II digital camera running analySIS 3.0 software.

2.4.2 Dynamic Light Scattering

Theory

DLS is frequently used to assess the hydrodynamic diameter of micro- and nanometre-sized particles in aqueous suspensions [16]. In this technique, the particle size is inferred from the extent of Brownian motion the particles display: small particles experience a higher degree of Brownian motion compared to larger ones. The motion of the particles is assessed using a laser beam which is directed through the sample. The light from the beam is scattered by the particles, such that a scattering pattern of fluctuating intensity can be observed using a detector at a particular angle to the incoming beam (Fig. 2.9a). The temporal change of this intensity fluctuation is observed, and a correlation function calculated. For large, slow moving particles, this pattern will change more slowly, and thus the rate of decay of the correlation function will be smaller, compared to smaller, fast moving

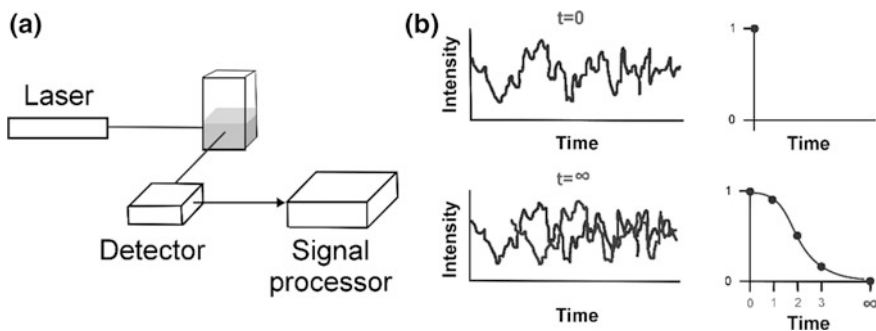


Fig. 2.9 Schematic of a dynamic light scattering experiment. **a** A sample is illuminated with laser light and the intensity pattern that is created by scattering from particles in the sample is observed with a detector. **b** Due to particle movement, the intensity pattern changes over time. Correlation of the pattern at a given time ($t = 1, 2, 3, \dots$) with the pattern at the start of the measurement ($t = 0$) gives rise to a correlation function, from which the hydrodynamic radius can be calculated

particles (Fig. 2.9b). Thus, the correlation function gives an indication of the particle's speed due to Brownian motion, which in turn relates to the particle size [17]. This relationship is described by the Stokes-Einstein equation:

$$d = \frac{k_B \cdot T}{3 \cdot \pi \cdot \eta \cdot D} \quad (2.3)$$

where d is the hydrodynamic diameter of the particles, k_B is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the medium in which the particles are suspended, and D is the diffusion coefficient, which can be calculated from the correlation function. By collecting several measurements for any one sample, a mean particle size, the **z-average**, and an estimate of the width of the size distribution, the **polydispersity index**, can be calculated.

Although this technique is very simple and powerful, care must be taken in the interpretation of the results, particularly if comparisons between samples are to be made. Because the result is determined through assessing particle motion, factors that influence this motion (e.g. temperature, ionic strength of the solution) can also influence the result and therefore need to be kept constant. It also needs to be considered that larger particles scatter light more strongly than small articles, such that a small amount of larger particles in a mixture of sizes can dominate the signal and therefore the calculated z-average. It is advisable to reduce contamination of the sample with large particles, such as dust and aggregates, by filtering or centrifuging the sample prior to DLS measurements.

Method

Dynamic light scattering (DLS) measurements were performed on a ZetaSizer Nano-ZS (Malvern Instruments, UK). All samples were suspended in 20 mM phosphate buffer at room temperature and pH 7, and adjusted to a protein concentration of approximately 1 mg mL⁻¹ before passing them through a filter of 220 nm pore size. 1 mL of each solution was placed in folded capillary tubes (Malvern Instruments, UK). A total of 13 to 15 measurements was collected for each sample.

2.4.3 Zeta Potentiometry

Theory

Charged particles in solution are surrounded by an electrical double layer of ions present in the solvent. In a dynamic particle system, ions are only retained within a theoretical plane of hydrodynamic shear, and the potential energy at this plane is referred to as the zeta potential (Fig. 2.10a) [18]. Particles with predominantly positive charges in their surface have a positive zeta potential, and negatively charged particles a negative one. The zeta potential is determined by measuring the electrophoretic mobility of a charged particle in an applied electric field (Fig. 2.10b). A laser is used to irradiate a sample and the scattered light is collected

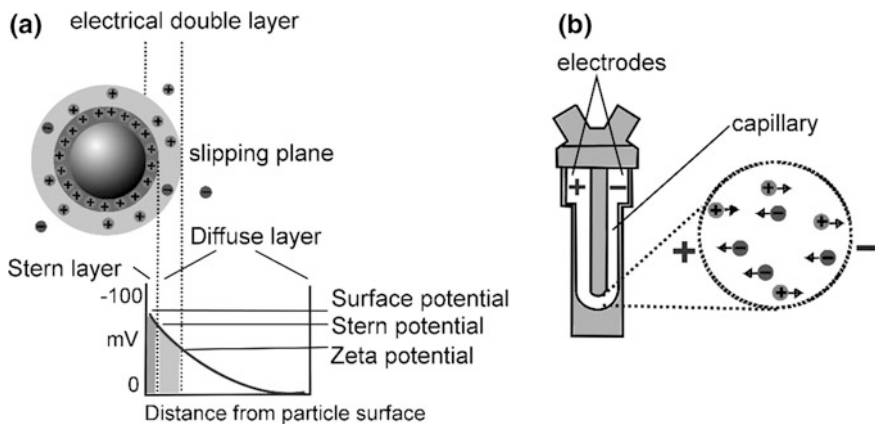


Fig. 2.10 The zeta potential of a charged particle. **a** Electrical double layer surrounding a charged particle in an electrolyte solution. **b** The folded capillary tube used to measure the electrophoretic mobility of charged particles in an applied electric field, from which the zeta potential can be calculated

as a function of time. The electrophoretic mobility is determined by comparing the phase of the scattered light with the phase of a reference beam [19]. The Henry equation relates electrophoretic mobility, EM , of a particle in a liquid medium to its zeta potential, ZP :

$$EM = \frac{2 \cdot \varepsilon \cdot ZP \cdot f(ka)}{3 \cdot \eta} \quad (2.4)$$

where ε is the dielectric constant of the medium, $f(ka)$ is the Henry's function, and η is the viscosity of the medium. The Henry's function describes the ratio of the particle's radius to the thickness of the electric double layer surrounding it. In general, either 1.5 or 1.0 are used as approximations of $f(ka)$. For measurements of particles suspended in aqueous media with moderate electrolyte concentration (as is the case in this work), 1.5 is used for $f(ka)$, and is referred to as the Smoluchowski approximation.

The most important factor that affects zeta potential is the pH of the solution, because it influences surface charge through protonation/deprotonation of functional groups. Therefore, zeta potential values must be quoted with the pH at which they were measured, otherwise they become an almost meaningless number.

Method

Zeta potential measurements were performed on a ZetaSizer Nano-ZS (Malvern Instruments, UK). Samples were suspended in 20 mM phosphate buffer at pH 7, and the protein concentration was adjusted to approximately 1 mg mL^{-1} and passed through a 220 nm filter. 1 mL of sample solution was placed in folded capillary tubes, and 10 to 12 measurements were performed on each sample.

2.4.4 Mass Spectrometry

Theory

Mass spectrometry is a technique to determine the molecular mass of large biomolecules, as well as small organic molecules. The measurements rely on the ionisation of the sample molecules, followed by their extraction into an analyser region, in which they are separated according to their mass-to-charge ratio (m/z). A detector monitors the ion current from the separated ions, and the intensity of the ion current can be plotted against m/z values to yield an m/z spectrum. This way, the number of components in the sample, and the molecular weight and relative abundance of each component can be determined.

In this work, matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry was used. This is a common technique to analyse samples of high molecular mass, such as proteins [20]. The sample is mixed with an excess of matrix compound (e.g. 2,5- dihydroxybenzoic acid) in a volatile solvent (e.g. methanol) that evaporates to yield a solid, crystalline matrix. This matrix strongly absorbs incident laser light, which results in thermal ablation. The sublimating matrix carries ionised sample molecules into the gas phase, where they are all accelerated to the same kinetic energy using an applied electric field. Accelerated ions are injected into a time-of-flight analyser, in which they are separated according to their m/z by measuring the time it takes for ions to move through a flight tube (heavier ions moving slower than lighter ones) (Fig. 2.11) [21]. The m/z scale can be calibrated using a sample of known molecular mass. MALDI spectra are relatively easy to interpret, because the gentle ionisation

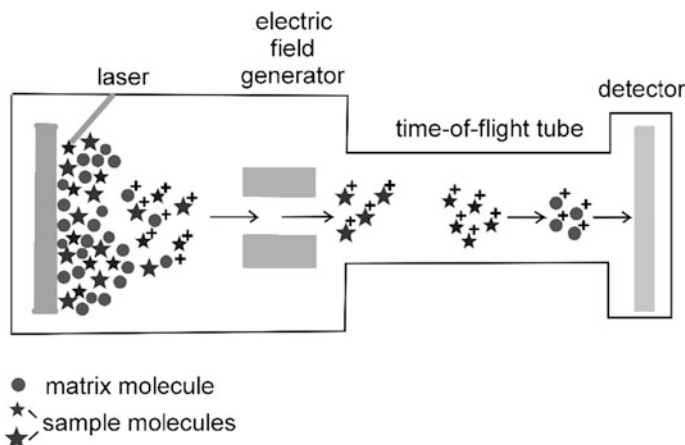


Fig. 2.11 Schematic of a MALDI-TOF experiment. The sample is embedded in a matrix, which strongly absorbs incident laser light. This causes thermal ablation of matrix and sample molecules, resulting in their ionisation and sublimation into the gas phase. An applied electric field accelerates the ions to the same kinetic energy. Accelerated ions are injected into a time-of-flight tube where they are separated according to their mass

method results in the generation of predominantly singly charged ions regardless of the molecular mass. This avoids multiple traces of the same molecule due to multiply charged sample ions.

Method

The MALDI-TOF experiment was performed on ApoF and cat-ApoF samples, instead of mineralised MF and cat-MF samples, because the mineralised protein has an exceedingly high molecular mass (over 900 kDa) due to the metal core. ApoF and cat-ApoF were dissolved in an equivalent volume of 20 mg mL⁻¹ of 2,5-dihydroxybenzoic acid in methanol. Samples of 2 µL were pipetted onto a steel plate and left to air dry and analysed in a Bruker UltrafleXtreme (Applied Biosystems, UK) running FLEX Control software (Bruker, USA).

2.4.5 Inductively-Coupled Plasma Optical Emission Spectroscopy

Theory

Inductively-coupled plasma optical emission spectroscopy (ICP-OES) is frequently used to quantify the elements present in a variety of samples, such as nanoparticles, drinking water, agricultural and biological samples [22]. This method relies on the fact that vaporised atoms can be excited by an inductively-coupled argon plasma and emit photons of a characteristic pattern of wavelengths as they return to the ground state. The wavelengths in the emitted spectrum are determined by the energy level structure of the atoms, thus, they can be used to identify the elements from which they originated. The amount of emitted light is proportional to the amount of atoms present in the sample, and by introducing known concentrations of analyte atoms, a calibration curve can be generated. Using ICP-OES, up to 70 elements can be detected simultaneously at low parts per billion (ppb) detection limits [22].

Method

MF samples were mixed with 50% (v/v) nitric acid to dissolve the metal atoms, and then diluted with water to reduce the acid concentration. The aqueous sample was introduced as an aerosol into the central channel of an argon plasma in a 710 ICP-OES instrument (Agilent, UK). To quantify the amounts of iron and cobalt in the MF samples, optical emission was measured at specific wavelengths characteristic for the emitted spectrum of these elements (approximately 255 nm for iron and 233 nm cobalt). Five calibration samples of known iron and cobalt concentrations were analysed alongside the MF samples. The protein concentration in the MF samples was determined using the Bradford assay (see Sect. 2.2.4), such that iron content per protein unit could be calculated.

2.4.6 SQUID Magnetometry

Theory

SQUID magnetometry uses a superconducting quantum interference device (SQUID) for the detection of magnetic moments in liquid or solid samples. It is a very sensitive technique, which allows detection of magnetic moments as small as 10^{-11} Am^2 [23]. The magnetic moment can be measured as a function of an applied field or an applied temperature. Hence, it is a common technique used to characterise important parameters of magnetic nanoparticles, such as saturation magnetisation and magnetic susceptibility. Both can be determined by measuring the magnetic moment as a function of an applied magnetic field. The blocking temperature can be estimated by measuring the magnetic moment as a function of the applied temperature. A measurement is performed by moving a sample through superconducting pick-up coils, by which a current is induced in the coils (Fig. 2.12a). This is converted into a voltage signal by the SQUID, with the amplitude of the generated voltage curve being proportional to the magnetic moment of the sample (Fig. 2.12b) [23, 24].

Method

Saturation magnetisation and magnetic susceptibility of MF and cat-MF were measured by performing a field sweep over $\pm 2 \text{ T}$ at 300 K using a SQUID magnetometer (Magnetic Property Measurement System, MPMS, Quantum Design, USA). A 0.2 mL tube (Corning, UK) and a drinking straw were used as a sample holder. The contribution of the sample holder to the overall sample moment was

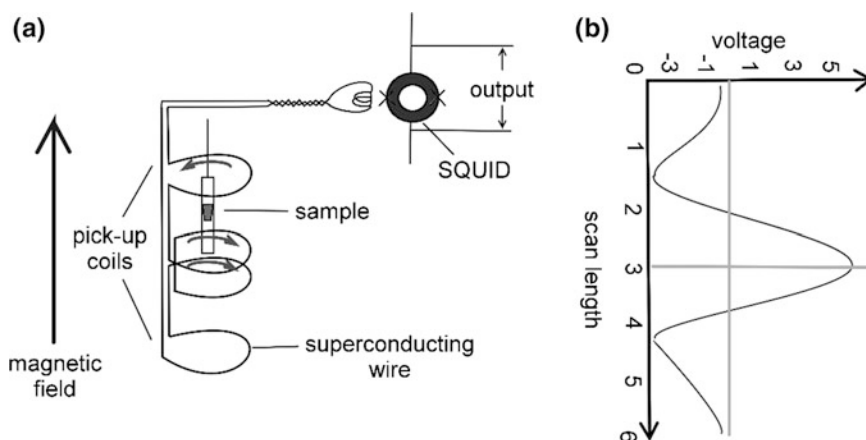
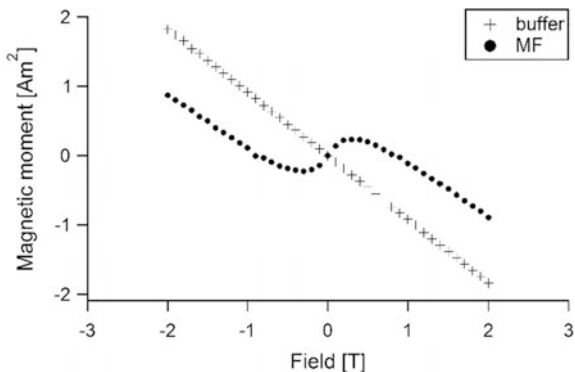


Fig. 2.12 Detection of small magnetic moments using a SQUID magnetometer. **a** A sample's magnetic moment in an applied magnetic field is measured by moving it through pick-up coils made from superconducting wire. The induced current is converted to a voltage signal using a superconducting quantum interference device (SQUID). **b** The voltage output as a function of sample position in the pick-up coils. The magnetic moment can be calculated from this voltage curve

Fig. 2.13 Assessing the diamagnetic background. The diamagnetic contribution to the overall MF sample moment can be mainly attributed to the phosphate buffer



measured separately and the background-corrected sample moment was determined as described in Appendix B. Briefly, a corrected voltage curve for MF/cat-MF was computed by subtracting the sample holder curve from the curve recorded for the sample and the sample holder together using a MATLAB (MathWorks, USA) script written by Dr. Carsten Putzke (University of Bristol). The best fit for this curve and the magnetic moment were found using equations supplied by the manufacturer.

The diamagnetic background from the aqueous solvent (50 mM phosphate buffer) was measured separately and found to be a major contributor to the diamagnetic response of the MF/cat-MF sample, particularly in high fields (Fig. 2.13). To correct for this diamagnetic contribution, a linear slope was subtracted from the magnetic moment of the sample.

Superparamagnetic behaviour was confirmed by fitting a Langevin function to the measured data points:

$$m(B) = \mu * n * L(x) \quad (2.5)$$

where $m(B)$ is the magnetic moment of the sample measured as a function of the applied field B , μ is the magnetic moment of an individual nanoparticle, n is the number of nanoparticles in the sample, and $L(x)$ is the Langevin function:

$$L(x) = \coth(x) - x^{-1} \quad (2.6)$$

with

$$x = \frac{\mu B}{k_B T} \quad (2.7)$$

where k_B is the Boltzmann constant and T is the absolute temperature. The saturation magnetisation is given by $\mu * n$. Magnetic susceptibility was calculated by determining the slope of the linear part of the magnetisation curve in the low field region (up to ± 0.3 T).

The blocking temperature was determined by cooling the sample from 120 K to 6 K in zero field, then applying a field of 0.01 T and measuring the magnetic moment of the sample at intervals of 1 K up to 25 K, and of 2 K up to 120 K.

2.4.7 Magnetic Resonance Imaging

Theory

When placed inside the magnetic coil of a magnetic resonance imaging (MRI) scanner, the magnetic moments of hydrogen nuclei (protons) in aqueous solutions align with the external magnetic field generated by the coil. The magnetic moments of these protons arise from their nuclear spins, which precess around the axis of the external field at a characteristic frequency, the so called Larmor frequency. The sum of all these spins results in a net magnetisation (M_0) that is aligned with the external field but of a much smaller magnitude (of the order of μT) (Fig. 2.14a). While the magnetisation is at equilibrium (lying parallel to the external field), it is not possible to measure it directly. However, radiofrequency (RF) pulses that are applied at the Larmor frequency (the protons' resonance frequency) can impart energy on the protons that flip the orientation of their spins 90° out of their longitudinal alignment with the external field into the transverse plane. Detector coils that are sensitive to only the transverse magnetisation can then pick up this magnetic resonance (MR) signal [25]. Thus, M_0 becomes a significant signal that can be recorded. This is done by creating echoes, which will be discussed below. When the RF pulse is removed, the protons can relax back to their initial state of equilibrium. There are two relaxation mechanisms, spin-spin and spin-lattice relaxation [26]. During spin-spin relaxation, neighbouring protons interact such that their precession frequencies gradually de-phase, and thus the MR signal measured in the transverse plane decays exponentially (Fig. 2.14b, top). Therefore, spin-spin relaxation is also called transverse relaxation. During spin-lattice relaxation, the protons transfer the energy they received from the RF pulse to the surrounding environment (the lattice) and thus gradually return to their equilibrium state, recovering their initial longitudinal magnetisation (Fig. 2.14b, bottom). Therefore, spin-lattice relaxation is also called longitudinal relaxation. When imaging humans or animals with MRI, the transverse and longitudinal relaxation times depend on the composition of the tissues, and each tissue type has a set of characteristic time constants associated with those processes. T_1 describes the time it takes for the spins to recover 63% of their longitudinal magnetisation, while T_2 describes the time it takes for the transverse magnetisation to drop to 37% of its initial value.

Both T_1 and T_2 can be influenced by introducing contrast agents, which can aid MRI based diagnoses [27]. The effect of contrast agents on relaxation times of individual tissues is described using relaxation rates, which are the inverse of the relaxation times, $R_1 = T_1^{-1}$ and $R_2 = T_2^{-1}$. For contrast agents, a specific relaxivity rx ($x = 1, 2$) is defined, which describes how much they change T_1 and T_2 per molar concentration.

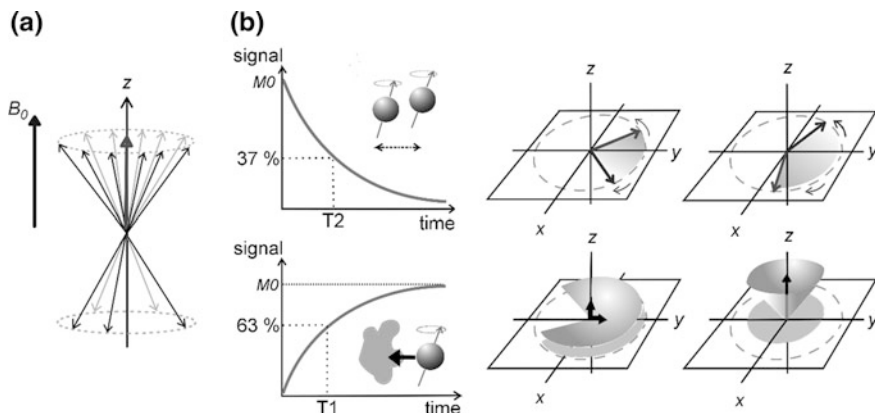


Fig. 2.14 MRI relaxation mechanisms. The nuclear spins of protons in an applied field which precess around the axis of the external field (B_0) at the Larmor frequency. The sum of all spins results in a net magnetisation (M_0) that is aligned with B_0 (blue arrow). A radiofrequency (RF) pulse that is applied at the Larmor frequency can impart energy to the protons and flip the orientation of their spins 90° out of their longitudinal alignment with the external field into the transverse plane. **b** Relaxation mechanisms after the RF pulse is removed. Top: during spin-spin relaxation, neighbouring protons interact such that their precession frequencies gradually de-phase (illustrated by a “fanning out” of the arrows). Thus the MR signal measured in the transverse plane decays exponentially. Bottom: during spin-lattice relaxation, the protons transfer the energy they received from the RF pulse to the surrounding environment and thus gradually recover their initial longitudinal magnetisation

As mentioned earlier, the MR signal and its temporal change can be measured by creating echoes. Two types of echoes are mainly used in MRI: gradient echo and spin echo. In a spin echo sequence, proton spins are left to de-phase for a certain amount of time after the 90° pulse. Then, a pulse is applied that flips all spins through 180° about the y axis, reversing their phase angles. This means that after the same time, all spins will re-phase to form a spin echo (Fig. 2.15). The echo height, and thus signal intensity, will mainly depend on the transverse relaxation time T2. Therefore, this is a useful sequence to measure T2 [25].

The gradient echo sequence also starts with an RF excitation pulse, usually with a flip angle smaller than the 90° used in the spin echo sequence. Instead of letting the spins de-phase naturally as in the spin echo, a negative magnetic gradient is applied immediately after the excitation pulse, which causes rapid de-phasing of the spins. After the negative gradient, a positive gradient is applied, which reverses the magnetic field gradient. This means that spins precessing at low frequencies due to their position in the gradient now precess at a higher frequency, and vice versa. Therefore, the spins start to re-phase and eventually all come back into phase along the y-axis, forming the gradient echo. However, the positive gradient only compensates for the de-phasing caused by the initially applied negative gradient. It does not re-focus the spins that have de-phased due to magnetic field inhomogeneities. Therefore, this imaging mode is very useful to image contrast enhancement caused

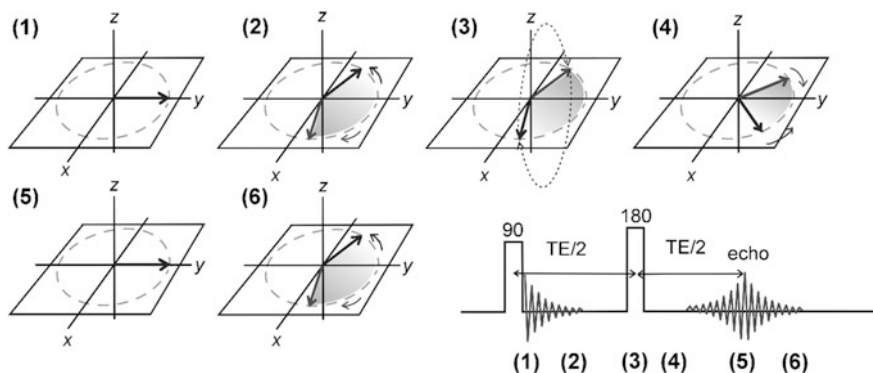


Fig. 2.15 Schematic of a spin echo sequence. An RF pulse flips the magnetisation of the protons into the transverse plane (1), and when the pulse is removed the spins de-phase (2). Another RF pulse is applied that flips all spins through 180° about the y axis, reversing their phase angles (3). As a result, spins start to re-phase (4) and eventually come into phase again (5) before de-phasing again (6)

by SPIONs, because SPIONs behave like small magnets in the tissue creating significant magnetic field inhomogeneities.

To measure T1 a series of inversion-recovery (IR) spin echo sequences with varying inversion delay times can be used [28]. IR sequences start with a 180° pulse that inverts the magnetisations of the spins, followed by a delay time (TI) during which T1 recovery occurs. When the 90° pulse is applied, which flips the spins in the transverse plane for detection, some of the signals may still be negative (inverted), resulting in a mixture of positive and negative echoes that are represented as light and dark voxels in the image. The repetition time (TR) between IR sequences should be at least five times the longest T1 to allow relaxation to the initial equilibrium magnetisation (M_0) after the 90° pulse (Fig. 2.16).

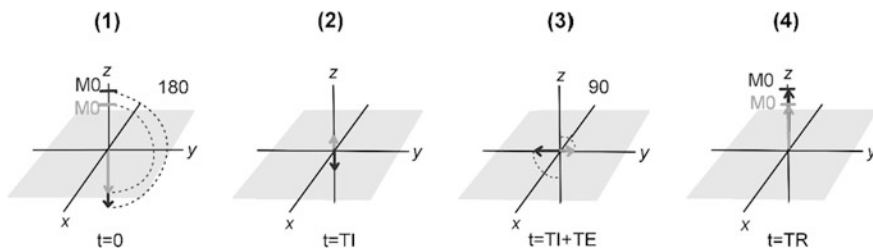


Fig. 2.16 Schematic of an inversion-recovery spin echo sequence. An initial 180° pulse inverts the magnetisations of the proton spins (1) (the two arrows represent spins in two different environments). During the delay time $t = TI$, T1 recovery occurs (2). When the 90° pulse is applied some of the signals may still be inverted, resulting in a mixture of positive and negative echoes (spatially distributed across the different environments) (3). The repetition time, TR, must always be at least five times the longest T1 to allow full relaxation (4)

Method

To determine specific relaxivities of MF and cat-MF, T1 and T2 were measured for protein concentrations ranging from 3 μM to 0.05 μM in phosphate buffered saline (PBS). 200 μL of each solution were placed in tubes of a PCR plate (Corning, UK) embedded in a 1.5% (w/v) carrageenan gel. T1 was determined using a series of IR spin echo sequences with varying TI. A TR of 6000 ms and an echo time of 9.6 ms was chosen. Signal intensity after the following TI was recorded: 50, 150, 250, 350, 450, 650, 850, 1250, and 2700 ms (slice thickness: 3 mm, field of view: 12 cm). The signal intensity, $S(TI)$, at each TI is given by:

$$S(TI) = M_0 \left[1 - 2 \exp\left(-\frac{TI}{T_1}\right) \right] \quad (2.8)$$

To measure T2, a series of spin-echo sequences was used and the signal intensities were measured after the following TE: 10, 20, 30, 50, 80, 100, 160, 240, 480 ms (slice thickness: 3 mm, field of view: 12 cm. Because transverse relaxation occurs faster than longitudinal relaxation, a shorter repetition time was chosen (TR = 3500 ms). The signal intensity, $S(TE)$, at each TE is given by:

$$S(TE) = M_0 \exp\left(-\frac{TE}{T_2}\right) \quad (2.9)$$

Signal intensities in the generated greyscale images were determined by region of interest (ROI) image analysis using a program written in Python (Python Software Foundation, Netherlands) by Ronald Hartley-Davies (University Hospitals Bristol NHS Foundation Trust). ROIs were determined by identifying the circular regions in the image, which represented the diameters of the PCR tubes containing the samples. Mean intensity of the pixels within the circular regions calculated by

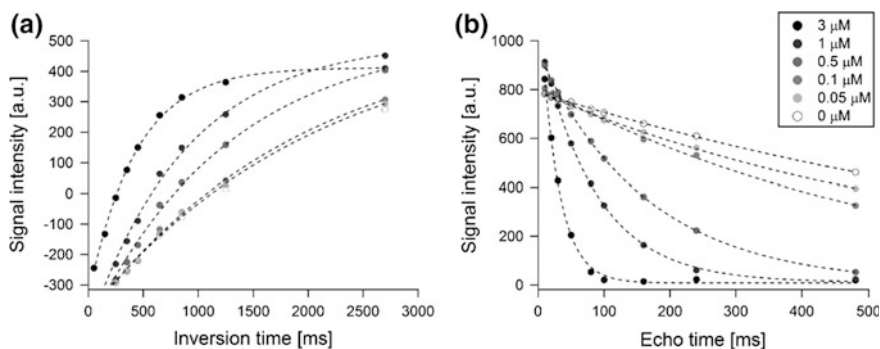


Fig. 2.17 Measuring T1 and T2 of MF. **a** A series of inversion recovery spin echo sequences with varying inversion times was performed for each MF concentration, and signal intensities were measured after each inversion time. **b** A series of spin echo sequences was performed and signal intensity was measured after varying echo times. The same data were also recorded for cat-MF

the software were plotted against the TI or TE, respectively, and T1 and T2 were determined by fitting exponential curves to the signal intensities according to Eqs. 2.8 and 2.9 (Fig. 2.17). The calculated T1 and T2 values were then plotted against the molar iron concentration present in the samples as determined by ICP-OES. The slope of the linear fit was determined to be the relaxivity, r_1 and r_2 , respectively.

2.5 Magnetoferritin Synthesis

Cobalt-doped MF was synthesised from commercially available horse spleen ApoF using an established protocol [29, 30]. Synthesis was performed in a double-jacketed reaction vessel at 65 °C containing 75 mL of deoxygenated 50 mM HEPES buffer (pH 8.6) with 3 mg mL⁻¹ ApoF. The vessel was kept under a nitrogen atmosphere to restrict oxidation of the metal precursors. 30.3 mL of 25 mM ammonium iron sulphate hexahydrate, containing 2.5% (w/v) cobalt sulphate heptahydrate was added at a rate of 0.15 mL min⁻¹ with a syringe pump (Titrand 907, Metrohm). Controlled oxidation of the metal precursors inside the ApoF cavity was mediated by adding an equivalent volume of an 8.33 mM hydrogen peroxide solution at the same rate. Fresh metal precursor and hydrogen peroxide solutions were injected three times at 65 min intervals. The initially colourless ApoF solution adopted a dark brown colour upon nanoparticle mineralisation, and after the final reagent addition it was left to mature for 15 min before 1.5 mL of 1 M sodium citrate were added to chelate free metal ions in the solution. Large precipitates were removed by centrifuging the sample for 30 min at 4350 × g and then passing the supernatant through a 220 nm syringe filter. The protein was purified by anion-exchange chromatography (see Sect. 2.3.1) to remove nanoparticles not enclosed in the protein cavity, followed by size-exclusion chromatography (see Sect. 2.3.2) to isolate protein monomers. Protein concentration was determined using a Bradford assay (see Sect. 2.2.4).

To compare the effect of cobalt-doping on the magnetic properties of MF, un-doped MF was also synthesised following the procedure described above, with the exception that the iron precursor was added without the addition of cobalt sulphate.

2.6 Magnetoferritin Cationisation

For cationisation, *N,N*-dimethyl-1,3-propanediamine (DMPA) was coupled to aspartic and glutamic acid residues on the MF surface using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) as described previously [31, 32]. Solutions of DMPA were adjusted to pH 7 using HCl, before drop-wise addition of MF or ApoF. After an equilibration period of 3 h, the

coupling reaction was initiated by adding EDC and adjusting the pH to 5 using HCl. The solution was continuously stirred for up to 24 h and then filtered through a 220 nm syringe filter to remove any precipitates. The solution was extensively dialysed (Medicell dialysis tubing, 12–14 kDa MWCO) against 4 L of 50 mM phosphate buffer (pH 7) containing 50 mM NaCl to yield stable solutions of cat-MF and cationised ApoF (cat-ApoF).

2.7 Characterisation of MF and cat-MF

To determine the size of MF and cat-MF transmission electron microscopy (TEM) and dynamic light scattering (DLS) were used (see Sect. 2.4.2). Core sizes of MF nanoparticles were determined by image analysis of unstained samples, and the diameters of MF and ca-MF (core and protein shell) were determined by image analysis of stained samples, both by employing Image J (National Institute of Health, USA) software. The length of the scale bar in the recorded TEM images was converted to a pixel measure. A mean threshold was applied to clear the image from debris, which left discrete black and white images of the nanoparticles that was subjected to particle analysis, from 0–infinity size and 0–1 circularity. This yielded a list of particle sizes, for which the mean (m_x) and standard deviation (s_x) were calculated using Excel (Microsoft Co., USA) as follows:

$$m_x = \frac{1}{n} \sum_{i=1}^n x \quad (2.10)$$

and

$$s_x = \sqrt{\frac{1}{n-1} \sum_{i=1}^n (x - m_x)^2} \quad (2.11)$$

where n is the sample size and x the observed value.

The composition of the nanoparticle core was determined using inductively-coupled plasma optical emission spectrometry (ICP-OES) (see Sect. 2.4.5). Cationisation efficiency of covalent DMPA coupling to the MF surface was assessed with zeta potentiometry (see Sect. 2.4.3). For the assessment of the number of DMPA molecules conjugated to each ApoF subunit as a measure of cationisation efficiency, matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry was used (see Sect. 2.4.4). Characterisation of the magnetic properties of MF and cat-MF was performed using SQUID magnetometry and MRI (see Sects. 2.4.6 and 2.4.7).

2.8 Histology

2.8.1 *Fixing, Embedding, Sectioning and Dewaxing*

Theory

Tissues are prepared for histological staining and imaging through fixation, embedding and sectioning. Fixation preserves the tissue's micro-structure and embedding in a rigid matrix supports the tissue during thin sectioning, which in turn is required for microscopic observation. Tissues and stem cells used in this work were fixed with paraformaldehyde, which crosslinks proteins with methylene bridges, [33] and embedded in paraffin wax. Paraffin melts around 60 °C, such that the liquid can effectively infiltrate the tissue. Upon cooling, paraffin solidifies and tissues enclosed in this "block" can be sectioned using a microtome. Prior to staining, this wax needs to be removed by immersion into organic solvents, such as xylene.

Method

Cartilage constructs or hMSCs grown on fibronectin-coated polyglycolic acid (PGA) scaffolds (Biomedical Structures, USA) were fixed for 3 h in 4% (w/v) paraformaldehyde solution at room temperature. After that the samples were immersed in 100% ethanol and submitted to the Histology Services Unit (Medical Sciences, University of Bristol), where they were embedded in paraffin wax, cut into 5 or 10 μm sections and placed on Polysine microscope slides (VWR, UK).

Before staining, sections were de-waxed and re-hydrated by sequential immersion into xylene, 100% ethanol, 70% ethanol and finally dH_2O , each for two minutes.

2.8.2 *Haematoxylin and Eosin Stain*

Theory

Haematoxylin and eosin (H&E) are frequently used to stain the cell's nucleus purple and the cytoplasm pink, respectively [34]. To produce a functional dye, haematoxylin is oxidized to haematin, which in turn forms a cationic complex with metal ions, such as aluminium (III). The metal cation mediates association of the dye molecule with acidic species, like nucleic acids through a metal-acid bond [35]. Eosin on the other hand binds to basic amino acid residues, thus staining proteins in the cytoplasm and extracellular matrix [36].

Method

De-waxed tissue sections on microscope slides were immersed in Haematoxylin QS solution (Vector Laboratories, UK) for two minutes and excess stain was removed by washing in dH_2O . They were then placed in a 10 mg mL^{-1} eosin Y solution for five minutes and the excess stain was removed by immersing the slides in 70%

ethanol, followed by 20 s in 100% ethanol. Slides were left to air dry and mounted on glass cover slips (VWR, UK) using DPX mounting medium.

2.8.3 *Safranin O and Fast Green Stain*

Theory

Safranin O is a cationic dye molecule, which binds electrostatically to anionic species in the tissue. In particular, anionic sulphate groups in proteoglycans form stoichiometric complexes with individual safranin O molecules [37]. Healthy cartilage tissue is rich in proteoglycans, such that safranin O staining can be used as a measure of proteoglycan distribution in the extracellular matrix of tissue engineered cartilage constructs [38]. The cytoplasm of the cells in the tissue are often counterstained with Fast Green, which is an anionic dye that interacts with cationic amino acid residues in a similar fashion to eosin [39].

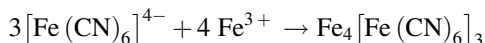
Method

De-waxed and re-hydrated tissue sections were immersed in a 1 mg mL⁻¹ Fast Green solution for four minutes. The excess stain was removed by washing in 1% (v/v) acetic acid solution. Then, sections were placed in a 5 mg mL⁻¹ Safranin O solution for six minutes. Excess stain was removed by dipping slides into 95% ethanol. Sections were de-hydrated in 100% ethanol for 20 s and then mounted onto glass cover slips with DPX mounting medium.

2.8.4 *Prussian Blue and Nuclear Fast Red Staining*

Theory

The Prussian Blue reaction is a histochemical method to stain iron deposits in cells and tissues, such as haemosiderin or ferritin [40, 41]. It is also widely used to visualise SPIONs internalised by cells [42]. The reaction involves a treatment with acidic solutions of ferrocyanides. Any ferric iron (Fe³⁺) in cells/tissues combines with ferrocyanide and results in the formation of ferric ferrocyanide, a bright blue pigment referred to as Prussian Blue:



Nuclear Fast Red is used as a red nuclear counterstain. Similar to haematoxylin, it is prepared in a solution containing excess aluminium ions, such that it becomes a cationic molecule that can bind to nucleic acids through a metal-acid bonds [43].

Method

De-waxed and re-hydrated sections were immersed in a 10% (w/v) potassium ferrocyanide solution mixed with an equal volume of 2 M hydrochloric acid for

20 min at room temperature. Slides were washed in dH₂O three times for five minutes. Cell nuclei were counter stained with a Nuclear Fast Red solution for five minutes and the excess stain was removed by rinsing with dH₂O. Sections were de-hydrated first in 70% then in 100% ethanol for 20 s and then mounted onto glass cover slips with DPX mounting medium.

2.8.5 Immunostaining of Collagen Type II

Theory

Immunohistochemistry makes use of antibodies to localise particular antigens in cells or tissues and stain those regions with high specificity [44]. However, during fixation of cells and tissues cross-linked proteins may mask these antigens. Therefore, they must first be retrieved and a common way of achieving this is to use proteolytic digestion. In the fixed cartilage tissue sections used here, proteoglycans and hyaluronic acid components of the extracellular matrix were digested with pronase and hyaluronidase, respectively. This exposed the collagen fibres present in the tissue, such that collagen type II epitopes (i.e. the regions of the antigen that the antibody binds to) could be targeted using an indirect immune-enzyme strategy (Fig. 2.18). In the first step, a primary antibody binds to collagen type II epitopes. Then, a biotinylated secondary antibody is added to bind to the primary antibody. Avidin is added to form strong electrostatic bonds with the biotin moiety of the secondary antibody. At the same time, avidin possesses binding sites for further biotinylated molecules, such as horseradish peroxidase. Therefore, a complex comprising avidin and biotinylated-horseradish peroxidase can bind to the secondary antibody. Horseradish peroxidase is an enzyme that can polymerise water

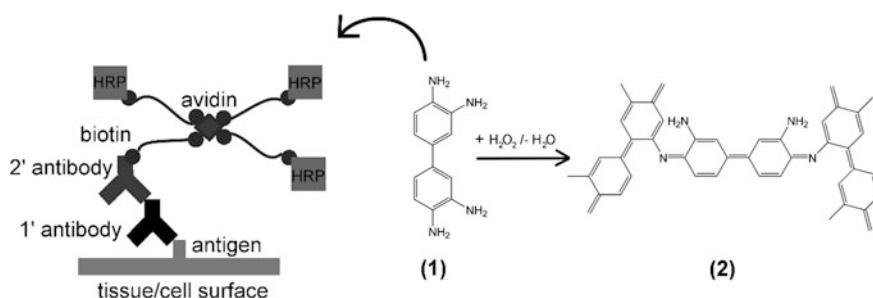


Fig. 2.18 Principle of the indirect immune-enzyme staining strategy. A primary (1') antibody binds to the epitope in the tissue or on the cell surface. A biotinylated secondary (2') antibody is added to bind to the 1'. Avidin is added and forms electrostatic bonds with the biotin moiety of the 2' antibody. A complex comprising avidin and biotinylated-horseradish peroxidase (HRP) can bind to the 2' antibody. Horseradish peroxidase catalyses the polymerisation of water soluble 3,3'-diaminobenzidine (DAB) tetrachloride (1) to an insoluble indamine polymer (2) through the reduction of hydrogen peroxide (H₂O₂)

soluble 3,3-diaminobenzidine (DAB) tetrachloride to an insoluble precipitate, [44, 45] which appears as a brown stain. Because the final step relies on peroxidase activity, any such innate activity in the tissue must be quenched to avoid unspecific staining. This is often achieved by incubation with hydrogen peroxide.

Method

De-waxed and re-hydrated sections were circled with a PAP pen (The Binding Site, UK) to create hydrophobic enclosures. 200 μL of a 10 mg mL^{-1} bovine testicular hyaluronidase solution was added to each section and the slides were incubated in a humidified chamber at 37 °C for 30 min. Slides were immersed in PBS for five minutes to remove excess enzyme. After that, 200 μL of a 2 mg mL^{-1} pronase solution (from *Streptomyces griseus*, Roche, Switzerland) was added to each section and the slides were incubated in a humidified chamber at 37 °C for 30 min. Slides were immersed in PBS for five minutes to remove excess enzyme. Then, 200 μL of 3% (v/v) hydrogen peroxide solution was added to each section, and they were incubated in a humidified chamber at room temperature for 5 min. Slides were immersed in PBS for five minutes to remove excess hydrogen peroxide. 200 μL of a blocking solution containing 30 mg mL^{-1} bovine serum albumin (BSA) solution was added and slides incubated in a humidified chamber at room temperature for 1 h. This was done to minimise unspecific binding of the primary antibody. Slides were washed by placing them three times for five minutes in fresh PBS.

Primary antibodies were prepared by dilution in **antibody diluent**, which consisted of 10 mg mL^{-1} BSA in Tris buffer (50 mM, pH 7.6) containing 150 mM NaCl and 0.05% (v/v) Tween 20. 70 μL of 1% (v/v) goat anti-type II collagen (Cambridge Biosciences, UK) or 5% (v/v) goat IgG isotype (Santa Cruz, USA) was added to each section and incubated in a humidified chamber at 4 °C overnight. Excess primary antibody solution was removed by a 15 min immersion in Tris buffer (50 mM, pH 7.6) containing 1 M NaCl, followed by two 15 min washes in Tris buffer. The secondary antibody, biotinylated Anti-Goat IgG (VECTASTAIN Elite ABC Kit, Goat IgG, Vector Laboratories, UK), was prepared as 0.5% solution in antibody diluent. 70 μL of the secondary antibody solution was added to each section and slides were incubated in a humidified chamber at room temperature for 1 h. Excess secondary antibody solution was removed by a 15 min immersion in Tris buffer (50 mM, pH 7.6) containing 1 M NaCl, followed by two 15 min washes in Tris buffer. 200 μL of ABC reagent containing the avidin and biotinylated horseradish peroxidase macromolecular complex (VECTASTAIN Elite ABC Kit) was added to the sections for 30 min. Excess reagent solution was removed by washing three times for 10 min in Tris buffer. 200 μL of diaminobenzidine tetrahydrochloride substrate (ImmPACT DAB Substrate Kit, Vector Laboratories, UK) was added to the sections for exactly ten minutes, and the excess substrate removed by rinsing the slides in dH_2O . Sections were de-hydrated by serial immersion into 70 and 100% ethanol for 2 min, and finally into xylene for 2 min to remove traces of the PAP pen. Slides were left to dry and then mounted on glass cover slips using DPX mounting medium.

2.9 Immunohistochemical Methods

2.9.1 *Cartilage Digestion*

Theory

Release and separation of extracellular matrix proteins, such as collagen and proteoglycans, from the PGA scaffold was achieved through digestion of engineered cartilage constructs using trypsin. Trypsin is a serine protease that cleaves C-terminal amide bonds of arginine and lysine residues [46]. An array of inhibitors was added during the digestion process to prevent proteolysis of the collagen type II epitope by other enzymes. Chymotrypsin activity was inhibited with tosyl phenylalanyl chloromethyl ketone (TPCK), [47] cysteine peptidases were inhibited using iodoacetamide, [48] aspartyl proteases were inhibited with pepstatin A, [49] and metalloproteinases were inhibited with EDTA [50]. After digestion, trypsin can be inactivated by heat-induced denaturation.

Prior to digestion, any residual liquid needs to be removed from the constructs to determine their dry weight. To this end, lyophilisation (or freeze-drying) was employed. This is a method for dehydrating aqueous samples without applying heat. Instead, samples are frozen in a low pressure chamber, which causes the sublimation of solvent molecules.

Method

Cartilage constructs were placed in 2 mL tubes (Eppendorf, UK) and lyophilised at $-50\text{ }^{\circ}\text{C}$ and low pressure ($< 0.05\text{ mBar}$) overnight using a ModulyoD freeze-drier (Fisher Scientific, UK). The dry constructs were weighed to find the dry mass of the whole cartilage construct. Dried constructs were digested in $50\text{--}350\text{ }\mu\text{L}$ of a 2 mg mL^{-1} digestion solution containing TPCK-treated trypsin supplemented with 200 mM iodoacetamide, 200 mM EDTA and 2 mg mL^{-1} pepstatin A at $37\text{ }^{\circ}\text{C}$ in a Vortemp 56 shaking incubator (Labnet, USA) overnight. A further $50\text{--}350\text{ }\mu\text{L}$ of the digestion solution was added and then tubes were incubated for 2 h at $65\text{ }^{\circ}\text{C}$ in a shaking incubator and vortex-mixed at 10 min intervals during the first hour. Samples were boiled for 15 min at $100\text{ }^{\circ}\text{C}$ to inactivate trypsin and then centrifuged for two minutes at $12300 \times g$. The supernatant containing the digested cartilage matrix components was removed and the undigested scaffold freeze-dried and weighed again to find the mass of the scaffold. The dry weight of the extracellular matrix was calculated by subtracting the dry weight of the undigested scaffold from the dry weight of the whole cartilage construct.

2.9.2 *Collagen Type II Assay*

Theory

Collagen type II is an integral part of the extracellular matrix component in cartilage [51]. Collagen type II was quantified by performing an inhibition enzyme-linked

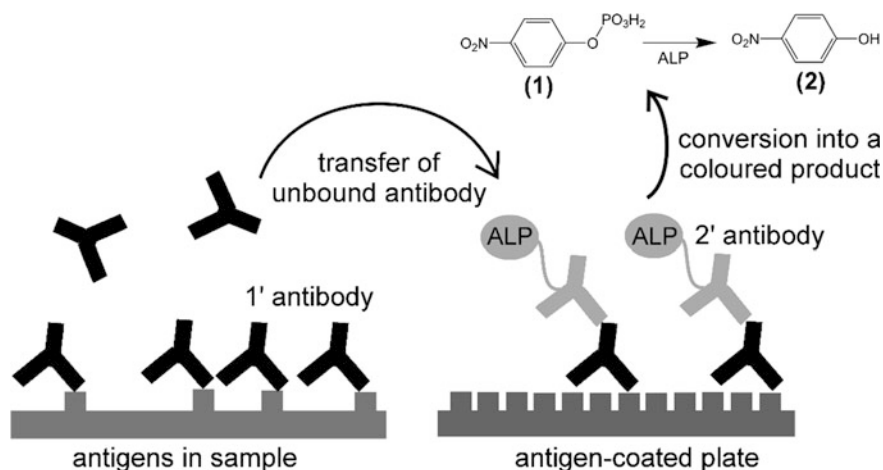


Fig. 2.19 Principle of the inhibition ELISA to quantify collagen type II. An excess of primary antibody is added to bind to antigens in the sample. The supernatant containing unbound primary antibody is transferred to a plate coated with collagen type II and binds to the antigens. An alkaline phosphatase (ALP)-conjugated secondary antibody binds to the immobilised primary antibody and catalyses the hydrolysis of colourless *p*-nitrophenyl phosphate (1) to yellow *p*-nitrophenol (2)

immunosorbent assay (ELISA). This assay was developed by Hollander et al, [52] who used the peptide CB11B (GKVGPSGA[Hyp]GEDGR[Hyp]GP[Hyp]GP) as a marker of denatured type II collagen. In the assay, a mouse antibody against type II collagen is incubated in excess with a range of CB11B standards and digested cartilage solutions. A set volume of each solution is transferred to a new plate with CB11B-coated wells. Any primary antibody not bound to collagen or peptide is available to bind the coated wells. Therefore, the more type II collagen present in the initial solution, the fewer antibody molecules bind to the wells of the second plate. A secondary antibody conjugated to alkaline phosphatase (ALP) is used to bind to the primary antibody. Alkaline phosphatase catalyses the conversion of colourless *p*-nitrophenyl phosphate substrate into a yellow *p*-nitrophenol product. This colour change can be quantified through absorbance measurements using a spectrophotometer. The absorbance signal from a series of peptide standards can be used to build a standard curve to estimate the quantity of type II collagen present in the digested cartilage matrix (Fig. 2.19).

Method

Collagen type II coated plates were prepared by coating the inner wells of a Costar Immulon-2 high binding 384 well plate (Appleton Woods) with 40 μL of heat-denatured type II collagen solution (Bioiberica, Spain). Plates were wrapped in cling film and left to incubate at 4 $^{\circ}\text{C}$ for three days. Plates were washed three times in PBS containing 0.1% (v/v) Tween 20 and left to dry. 50 μL of a 10 mg mL^{-1} BSA blocking solution was added to each well and incubated for 30 min at room

temperature. Plates were washed in PBS containing 0.1% (v/v) Tween, dried at 37 °C for 20 min and stored wrapped in cling film at 4 °C until use.

80 μL of a 10 mg mL^{-1} BSA blocking solution was added to the inner wells of a fresh Costar 384 well plate and incubated for 30 min at room temperature. Plates were washed in PBS containing 0.1% (v/v) Tween and left to air dry. Samples along with calibration standards of collagen II (Collagen Type II fragment, CB11B, Pepteuticals, UK; concentrations ranging from 0.5–6 $\mu\text{g mL}^{-1}$) were plated out in triplicate and incubated with a 0.17% (v/v) mouse-derived collagen type II antibody (COL2–3/4) at 37 °C overnight. After that, samples were transferred into the prepared collagen type II-coated plates for exactly 30 min at room temperature. The plates were then washed three times, and 10 μL of a 0.1% (v/v) solution of an ALP-conjugated goat anti-mouse secondary antibody (Cambridge Biosciences, UK) prepared in a 10 mg mL^{-1} BSA blocking solution was added to each well. The plates were sealed and incubated for 2 h at 37 °C. A disodium *p*-nitrophenyl phosphate tablet was dissolved in 10 mL of diethanolamine buffer, yielding a solution of approximately 0.5 mg mL^{-1} . After incubation with the secondary antibody, the plates were washed, 10 μL of the *p*-nitrophenyl solution was added to all wells and the plates left to incubate for 20 min at 37 °C. Then absorbance was measured at 405 nm using UV/Visible spectroscopy. The amount of collagen II was calculated from the mean absorbance of the CB11B peptide standards and expressed as a percentage of the extracellular matrix dry weight. The percentage of collagen II in cat-MF treated and untreated cartilage constructs was compared using the Mann-Whitney U-test in IBM SPSS Statistics version 21. A non-parametric test was chosen because of the small sample size, which made it difficult to confirm normality for the data sets. The Mann-Whitney U-test was employed because it is a useful test to compare differences between two treatment groups.

2.9.3 Glycosaminoglycan Assay

Theory

Glycosaminoglycans (GAG) are long unbranched polysaccharides containing repeating disaccharide units, and chondroitin sulphate is an abundant GAG in cartilage [53]. Chondroitin sulphate is an anionic molecule that forms an electrostatically bonded complex with the cationic dye 1,9-dimethylene blue (DMMB) [54]. Therefore, GAG content in engineered cartilage constructs can be determined using a DMMB-based assay. The binding of chondroitin sulphate causes a shift in the absorption maximum of DMMB, from 649 nm for the free molecule to 525 nm for the complex [55].

Method

20 μL of the digested cartilage construct matrix was added to a 96 well plate, alongside a series of chondroitin sulphate standards (5–50 $\mu\text{g mL}^{-1}$). 250 μL of a 160 $\mu\text{g mL}^{-1}$ dimethylmethyle blue solution was added to all wells and the

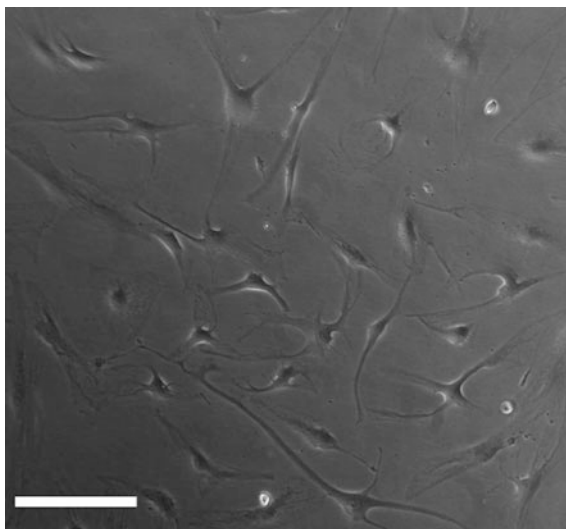
absorbance measured immediately at 530 nm using UV/Visible spectroscopy. The mean absorbance of the chondroitin sulphate standards were used to plot a standard curve and estimate the GAG concentration in the digested cartilage constructs, which was expressed as a percentage of the construct's dry weight. The percentage of GAG in cat-MF treated and untreated cartilage constructs was compared using the Mann-Whitney U-test in IBM SPSS Statistics version 21 (see Sect. 2.9.2 for rationale behind statistical test).

2.10 Mesenchymal Stem Cell Culture

2.10.1 Culture Conditions

Human mesenchymal stem cells (hMSC) were harvested from the proximal femur bone marrow of osteoarthritic patients undergoing total hip replacement surgery, in full accordance with Bristol Southmead Hospital Research Ethics Committee guidelines (reference #078/01) and after patient consent was obtained. All cell culture was performed in Hera Safe class II laminar flow cabinets (Kendro, Germany) and incubated in humidified Hera Cell 150 incubators (Kendro, Germany) at 37 °C and 5% carbon dioxide atmosphere. The cells were cultured as monolayers using 175 cm² flasks (Appleton Woods, UK) and 20 mL of culture medium, which was replenished every 3–4 days (Fig. 2.20). **Culture medium** consisted of Dulbecco's Modified Eagle's Medium (DMEM), containing 1000 mg L⁻¹ glucose, 10% (v/v) foetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin solution, 1% (v/v) glutamax solution (Life Technologies) and 5 ng mL⁻¹ freshly supplemented human fibroblast growth factor (PeproTech).

Fig. 2.20 Light microscopic image of human mesenchymal stem cells in culture (passage 6). Magnification 10×. Scale bar 100 µm



The hMSCs were inspected before each media change using bright field microscopy, and harvested and re-seeded (passaged) once they reached confluency. hMSCs were used for experiments up to passage 6.

2.10.2 Harvesting, Counting and Passaging hMSCs

Cells were harvested either for passage, cryopreservation or in preparation for an experiment. Culture medium was aspirated and cells washed with 20 mL PBS. 5 mL of trypsin/EDTA solution was added to the flask and incubated at 37 °C for five minutes. Cell detachment was confirmed using bright field microscopy, before addition of 10 mL of culture medium. The cell suspension was removed into 15 mL centrifuge tubes (Appleton Woods, UK) and centrifuged for five minutes at $524 \times g$. The supernatant was removed and the cell pellet re-suspended in culture medium. A 10 μ L sample of the cell suspension was placed in an AC1000 improved Neubauer haemocytometer (Hawksley, UK). For passage, hMSCs were re-seeded at a low cell density in 175 cm² flasks in 20 mL culture medium.

2.10.3 Freezing and Thawing hMSCs

Cells were harvested, counted, and samples containing 10⁶ hMSCs were centrifuged and the supernatant removed. The cell pellet was re-suspended in 1 mL of freezing medium, which consisted of two parts 10% (v/v) dimethyl sulfoxide in FBS, and one part of culture medium. The cell suspension was transferred into 1.5 mL cryovials (Fisher Scientific, UK) and placed in a Mr. Frosty freezing container (Fisher Scientific, UK) overnight at -80 °C. After that they were transferred to a Biorack 6000 refrigerator (Statebourne Cryogenics, UK), where they were stored in the vapour phase of liquid nitrogen (approximately -190 °C).

Cryopreserved hMSCs were thawed in a 37 °C water bath, transferred immediately into a 175 cm² flask containing 20 mL of culture medium and cultured as described in Sect. 2.10.1.

2.11 Stem Cell Labelling with cat-MF

This section describes the procedures carried out to investigate magnetic labelling of hMSCs with cat-MF. To assess labelling rapidity and efficiency, MACS was used. First, the characterisation of the MACS magnet is summarised, followed by the description of the MACS experiments. Then, the procedures to image hMSCs labelled with cat-MF using MRI, TEM and light microscopy are reported. Finally, an experiment to elucidate the labelling mechanism is described.

2.11.1 Characterisation of the MACS Magnet and Column

The magnetic field of a miniMACS separator magnet (Miltenyi Biotec) (Fig. 2.21a) was measured with a Gaussmeter and a transverse Hall Effect probe (Hall Effect Gaussmeter, Model 5170 F.W. Bell, Sypris Test & Measurement). The probe measured magnetic flux density B perpendicular to a sensing area of 0.38 mm in diameter, located 0.85 mm from the end of the rectangular probe rod (Fig. 2.21b).

The probe was placed in the MACS column cavity and B was recorded every millimetre along the entire length of the magnet from magnetic North to South (Fig. 2.22a). The magnetic field gradient was calculated from the linear portion of a plot of B as a function of position inside the cavity and found to be 57 mT/mm over the first and last 10 mm. In the middle of the cavity the field remained constant at approximately 500 mT over 10 mm (Fig. 2.22b).

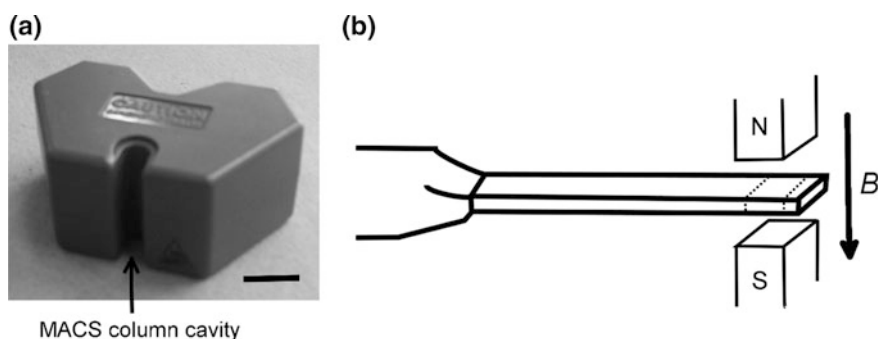


Fig. 2.21 **a** Photograph of the MACS magnet. Scale bar 1 cm. **b** Transverse Hall probe measuring magnetic flux density B perpendicular to a sensing area (dashed outline)

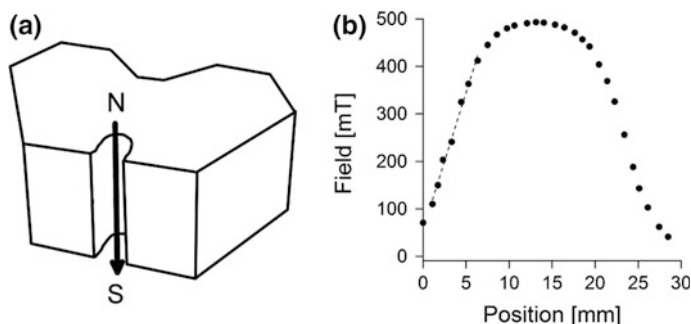


Fig. 2.22 Characterisation of the MACS magnet. **a** Schematic of the magnet, arrows indicate the direction of measurement. N and S: magnetic North and South, respectively. **b** Measurements of the magnetic field strength from magnetic North (position: 0 mm) to South. The dashed line indicates the linear fit used to determine the magnetic field gradient

The ferromagnetic matrix of the MACS columns used with this magnet was 20 mm long and extended between approximately 5 - 25 mm along the area measured in Fig. 2.22b. The diameter of the column was 4 mm and it fitted tightly into the width of the MACS column cavity. According to the manufacturer, the column matrix is composed of ferromagnetic beads with a diameter of 250 to 500 μm , coated in a polymeric material to protect cells from damage, and the space between the spheres is 100–200 μm . The flow rate in the column was measured to be approximately 0.5 mL min^{-1} .

2.11.2 *Magnetic Stem Cell Labelling: Time and Concentration Dependence*

To assess the rapidity of magnetic labelling with cat-MF, 150000 hMSCs from three different patients were seeded in culture medium into 25 cm^2 tissue culture flasks and left to adhere over night. Cells were washed with PBS and exposed to 1 mL of $0.5 \mu\text{M}$ cat-MF in PBS for 1 min to 6 h. After labelling, cells were washed with PBS and harvested from the flasks using trypsin/EDTA. Cell pellets were re-suspended in 0.5 mL magnetic separation buffer (0.5% (w/v) FBS and 2 mM EDTA in PBS) and magnetic-activated cell separation (MACS) was performed using MACS MS columns (Miltenyi BIOTEC) with a capacity to immobilise up to 10^7 magnetically labelled cells. First 0.5 mL of the sample in MACS buffer was loaded into the reservoir of the column. Once the reservoir was empty, 0.5 mL of MACS buffer was added three times (total volume 1.5 mL) to elute cells that were not magnetised sufficiently to be retained in the column. After that, the column was detached from the magnet, 1 mL of MACS buffer was added and pushed through the column using a plunger. This eluted the magnetised cells from the column matrix. Cell numbers in the magnetic and non-magnetic cell fractions were determined using an Improved Neubauer haemocytometer (see Sect. 2.10.2). To assess magnetisation efficiency, the fraction of magnetised cells, $M(\%)$, was determined as follows:

$$M(\%) = 100 \cdot \frac{n(M)}{n(M + NM)} \quad (2.12)$$

where $n(M)$ is the number of cells in the magnetic fraction and $n(M + NM)$ is the sum of the number of cells in the magnetic and non-magnetic fractions. Cells in the magnetic fractions were digested with 50% (v/v) nitric acid before iron content was determined using ICP-OES. The quantity of iron measured was normalised to the number of cells in the analysed fraction. The saturation value for cellular iron, Fe_{max} , and the time constant for iron uptake, k , were determined by fitting an exponential function to the experimental data over the 6 h time course:

$$Fe(t) = Fe_{\max} \left[1 - \exp\left(-\frac{t}{k}\right) \right] \quad (2.13)$$

where $Fe(t)$ is the cellular iron content at a particular time point and t is the incubation time in hours.

To assess the effect of cat-MF concentration on labelling efficiency, 150000 hMSCs from three different patients were seeded in culture medium into 25 cm² tissue culture flasks and left to adhere over night. Cells were washed with PBS and exposed to 1 mL of cat-MF concentrations ranging from 0.01 to 2 µM in PBS for 30 min. After labelling, cells were washed with PBS and harvested from the flasks. MACS and ICP-OES were performed as described above.

Finally, to confirm the effect of cationisation on labelling efficiency, 150000 hMSCs from three different patients were seeded in culture medium into 25 cm² tissue culture flasks and left to adhere over night. Cells were washed with PBS and exposed to 1 mL of MF concentrations ranging from 0.5 to 3 µM in PBS for 30 min. After labelling, cells were washed with PBS and harvested from the flasks. MACS and ICP-OES were performed as described above.

To calculate the minimum number of cat-MF particles needed to magnetise hMSCs sufficiently for retention in the MACS column, the magnitude of the magnetic force, F_m , acting on the magnetically labelled cells needs to compensate the drag force, F_d , experienced during the flow through the column. The magnetic force on a labelled cell can be approximated as:

$$F_m = N \cdot \mu \cdot \nabla B \quad (2.14)$$

where N is number of magnetic particles on the cell, μ is the magnetic moment of an individual magnetic nanoparticle (which can be determined from the Langevin function, see Eq. 2.5), and ∇B is the magnetic field gradient. The drag force can be described by:

$$F_d = 6 \cdot \pi \cdot \eta \cdot r_{cell} \cdot v \quad (2.15)$$

where η is the viscosity of the medium in which the cells are suspended (predominantly water), r_{cell} is the radius of the cell and v is the velocity of the cell as it's passing through the column.

2.11.3 MRI Imaging of Labelled hMSCs

800000 hMSCs were seeded in culture medium in 75 cm² tissue culture flasks and left to adhere over night. The cells were labelled with 3 mL of 0.5 µM of MF or cat-MF for 30 min. The MF or cat-MF supernatant was removed and cells were washed with PBS, harvested with trypsin/EDTA and counted. 750000 labelled cells were suspended in 200 µL of PBS and transferred into tubes of a PCR plate

embedded in a 1.5% (w/v) carrageenan gel and left to settle by gravity for 4 h prior to MRI imaging. T1 and T2 were determined as described in Sect. 2.4.7. Additionally, a gradient echo sequence was used with TR = 100 ms, TE = 10 ms, and a flip angle of 30° to image the labelled cells.

2.11.4 TEM Imaging of Labelled hMSCs

50000 hMSCs were seeded in culture medium into a 6 well plate and left to adhere over night. The cells were washed with PBS and exposed to 1 mL of 0.5 μ M cat-MF for 30 min. The cat-MF supernatant was removed and the cells were washed with PBS. The cells were either immediately fixed and stained for TEM imaging or cultured for one week before fixing and staining (see Sect. 2.4.1).

2.11.5 Prussian Blue Staining of Labelled hMSCs

1000000 hMSCs were seeded in culture medium into 75 cm² tissue culture flasks and left to adhere overnight. The cells were exposed to 3 mL of 0.5 μ M of cat-MF for 30 min. The cat-MF supernatant was removed, cells were washed with PBS and left in culture medium for 24 h. After that, hMSCs were harvested with trypsin/EDTA and counted. 300000 cells were suspended in 30 μ L of culture medium and loaded onto fibronectin-coated PGA tissue engineering scaffolds of 5 mm diameter and 2 mm thickness (Biomedical Structures, USA) and placed in agarose coated wells of a 24 well plate. Cells were left to attach to the scaffold over night, and then cultured for 6 h in culture medium. After that, the medium was removed and scaffolds fixed in 4% (w/v) paraformaldehyde over night at room temperature, dehydrated in 70% ethanol for 2 h and then submitted to the histology service of the School of Cellular and Molecular Medicine at the University of Bristol. Histological sections of 10 μ m thickness were stained with Prussian Blue and Nuclear Fast Red (see Sect. 2.8.4) and observed using an inverted light microscope at 40 $\times$ magnification (Leica DM IRB).

2.11.6 Long Term Retention of Magnetisation

To investigate the long term retention of magnetisation following labelling with cat-MF, 150000 cells were seeded in culture medium into 75 cm² tissue culture flasks and left to adhere over night. The cells were washed with PBS and exposed to

3 mL of 0.5 μM cat-MF for 30 min. The cat-MF supernatant was removed, the cells washed with PBS and then cultured for up to five weeks. Magnetisation and iron content of the cells were analysed at weekly intervals using MACS and ICP-OES.

To assess the long term retention of cat-MF for MR imaging, 800000 cells were seeded into 75 cm^2 flasks, left to adhere over night, and then labelled with 3 mL of 0.5 μM of cat-MF for 30 min. The cat-MF supernatant was removed, the cells were washed with PBS, and cultured in DMEM for either four days or one week before being harvested. 750000 labelled hMSCs were suspended in 200 μL of PBS and transferred into tubes of a PCR plate embedded in a 1.5% (w/v) carrageenan gel and left to settle by gravity for 4 h prior to MRI imaging using a gradient echo sequence with TR = 100 ms, TE = 10 ms, and a flip angle of 30°. T1 and T2 were also determined as described in Sect. 2.4.7.

2.11.7 *Elucidating the Labelling Mechanism*

To evaluate the contribution of active internalisation processes on cellular iron content 150000 hMSCs were seeded in culture medium into 25 cm^2 tissue culture flasks and left to adhere over night. hMSCs were exposed to 1 mL of 0.5 μM cat-MF for 5 or 30 min at 37 °C or 4 °C. Prior to incubation with cat-MF at 4 °C, cells were pre-cooled at 4 °C for 30 min in DMEM supplemented with 20 mM HEPES buffer. After each incubation period, the cat-MF supernatant was removed and the cells were washed, harvested and analysed using ICP-OES.

To test the hypothesis that anionic proteoglycans in the glycocalyx facilitate cat-MF uptake 150000 cells were cultured for four days in 25 cm^2 tissue culture flasks using DMEM supplemented with 80 mM sodium chlorate (NaClO_3), because NaClO_3 inhibits the synthesis of anionic functional groups on proteoglycans [56]. hMSCs were labelled with 1 mL of 0.5 μM cat-MF for 5 or 30 min at 37 °C before the cat-MF supernatant was removed and the cells were washed, harvested and analysed using ICP-OES.

Statistical analysis was undertaken for each time point separately (5 and 30 min). The cellular iron content values in hMSCs labelled under different conditions were compared using the Kruskal-Wallis test in IBM SPSS Statistics version 21. A non-parametric test was chosen because of the small sample size ($n = 3$), which made it difficult to confirm normality for the data sets. The Kruskal-Wallis test was used because more than two groups were compared. The statistics program also conducted pair-wise analysis as part of the procedure, which identified differences between groups.

2.12 Assessing Cell Viability After cat-MF Labelling

Theory

A common method to probe cell viability is the assessment of cellular metabolic activity. Cell metabolism results in the production of adenosine tri phosphate (ATP), which is the “energy currency” that is required for many processes in the living cell. A so-called electron transport chain is used to synthesis ATP, and thus, assessing the function of this vital process gives an indication of cell viability. Many commercially available biochemical assays probe metabolic activity by incubating cells with a dye that enters the cell and can be reduced by electrons from the electron transport chain. The formation of the reduced product can be quantified spectrophotometrically by measuring absorbance or fluorescence at a characteristic wavelength, and absorbance or fluorescence intensity are directly proportional to the number of metabolically active cells.

Among the most widely used dyes are tetrazolium salts, such as 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The dye is formulated into an assay solution that can be dissolved in culture medium. When the assay solution is added to the cells, the dye can access the intracellular space where it is reduced to formazan. This process is thought to be mediated by enzymes of the electron transport chain that generate reducing agents, such as nicotinamide adenine dinucleotide hydride (NADH). NADH can transfer its electrons to an electron transfer reagent (ETR), which is usually incorporated in the assay solution. Thus, the ETR is reduced and subsequently interacts with the tetrazolium compound, reducing it to a deeply coloured formazan product (Fig. 2.23a) [57]. Formazan has an absorbance maximum at 490 nm and can therefore be quantified by measuring absorbance at this wavelength. The measured absorbance values can then be compared between cells treated with a test compound and untreated cells to assess the impact of the test compound on cell viability.

Similarly, resazurin is a molecule that can be taken up by many cell types, and is used in the so-called Alamar Blue assay. If the cells are in a proliferative state, the reductive intracellular environment will lead to the reduction of resazurin to fluorescent resorufin using NADH (Fig. 2.23b) [58]. Fluorescence intensity can be measured upon excitation of the fluorescent compound at 570 nm and measuring emission at 590 nm. Again, the measured fluorescence intensity values can be compared between cells treated with a test compound and untreated cells to assess the impact of the test compound on cell proliferation. Both the MTS and the Alamar Blue assay measure similar endpoints, but the advantage of the Alamar Blue assay is that cells can be incubated with resazurin over prolonged periods of time, during which the formation of the resorufin product can be measured repeatedly. Therefore, it can be investigated whether adverse effects are transient or persistent. Both assays are sensitive to cell number and incubation time with the dye, therefore, both parameters were optimised for the hMSCs used here.

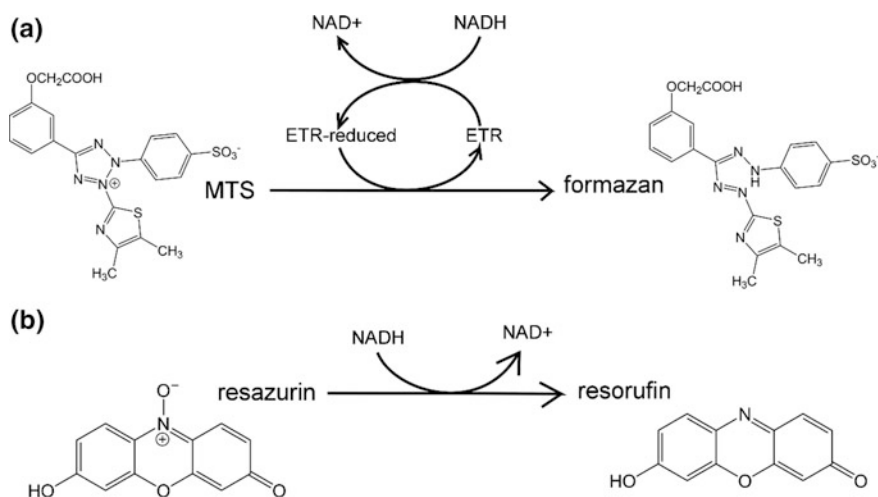


Fig. 2.23 Chemistry of viability assays. **a** The reduction of MTS to formazan through electron transfer reactions inside viable cells. An electron transfer reagent (ETR) is often added to the assay formulation, which acts as an intermediate electron acceptor and results in the formation of a soluble formazan compound. **b** Similarly, reduction of resazurin to fluorescent resorufin happens in the reductive environment of viable cells, mediated by nicotinamide adenine dinucleotide hydride (NADH)

2.12.1 Optimisation of the MTS and Alamar Blue Assay

Formation of the coloured products that are measured in the MTS and Alamar Blue assay is highly dependent on the number of cells present in the sample. Therefore, an appropriate number of cells needs to be chosen to ensure the validity of these assays. The ideal cell number can be determined by performing the assays on a variety of cell numbers and determining which cell numbers produce a signal that lies within the linear range of the assay.

A range of hMSC numbers between 1000 and 10000 was seeded in triplicate into 96 well plates and left to adhere over night. Culture medium was removed, cells washed with PBS and either incubated for 1 h at 37 °C with 100 μ L of phenol-free culture medium containing 20% (v/v) of MTS solution (CellTiter 96, Promega), or for 4, 24 and 48 h at 37 °C with 100 μ L of culture medium containing 10% (v/v) Alamar Blue solution (Serotec). Formation of reduced dye products was assessed by either measuring absorbance at 490 nm (MTS assay) or fluorescence intensity at excitation 570 nm and emission 590 nm (Alamar Blue assay) using a spectrophotometer.

For the MTS assay, a linear response was observed when the MTS reagent was incubated for 1 h with 1000 to 10000 hMSCs ($r^2 = 0.98$; Fig. 2.24a). Similarly, the fluorescence intensity of Alamar Blue increased linearly with cell number after 4 and 24 h incubation ($r^2 = 0.98$ and 0.99 , respectively). After 48 h, the fluorescence

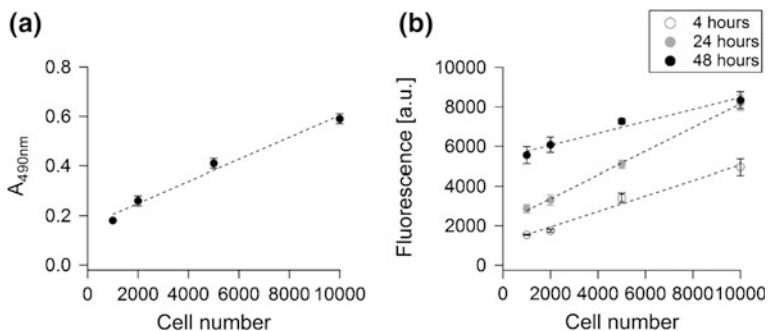


Fig. 2.24 Optimisation of cell viability assays. **a** Absorbance in the cell culture supernatants of 1000 to 10000 hMSCs exposed to MTS reagent for 1 h at 37 °C. **b** Fluorescence intensity (excitation 570 nm, emission 590 nm) in wells containing 1000 to 10000 hMSCs incubated with Alamar Blue reagent for 4, 24 and 48 h at 37 °C

intensity value recorded for 10000 cells was the same as the value recorded after 24 h (Fig. 2.24b). Therefore, it was concluded that a cell number below 10000 had to be chosen to obtain reliable results over a 48 h test period.

2.12.2 MTS and Alamar Blue Assay After Exposure to MF and cat-MF

For the **MTS assay**, 5000 cells from three different patients were seeded in culture medium in quadruplicate into wells of a 96 well plate, left to adhere over night, and exposed for 30 min to 125 μ L of MF or cat-MF at concentrations ranging from 0.01 to 3 μ M, or PBS only (untreated control). The supernatant was removed, cells washed with PBS and 100 μ L culture medium was added to each well. After 24 h, culture medium was removed, cells washed with PBS and incubated for 1 h at 37 °C with phenol-free culture medium containing 20% (v/v) of MTS solution. Formation of the reduced formazan product was measured at 490 nm using a spectrophotometer, and cell viability values of MF or cat-MF exposed cells (MF_{A490}) were normalised with respect to the untreated control (UTC_{A490}):

$$\%viability = \frac{MF_{A490}}{UTC_{A490}} * 100 \quad (2.16)$$

For the **Alamar Blue assay**, 2000 cells from three different patients were seeded in culture medium in quadruplicate into wells of a 96 well plate, left to adhere over night and exposed for 30 min to 125 μ L of MF or cat-MF at concentrations ranging from 0.01 to 3 μ M, or PBS only (untreated control). The supernatant was removed, cells washed with PBS and 100 μ L of culture medium containing 10% (v/v) Alamar Blue solution was added to each well. After 4, 24 and 48 h, fluorescence intensity

was measured at excitation 570 nm and emission 590 nm using a spectrophotometer. Cell viability values were normalised with respect to the untreated control as described in Eq. 2.16.

For **statistical analysis** of results obtained from the MTS and Alamar Blue assays, data were presented as mean $\pm$ standard deviation of three biological repeats (total $n = 12$). Statistical analysis was performed using IBM SPSS Statistics version 21. The means of groups treated with MF, cat-MF, and PBS (untreated control) were compared using Two-Way Analysis of Variance (ANOVA), in which effects of dose as well as surface functionalisation (unfunctionalised vs. cationised) were investigated, followed by a one-sided Dunnett t post hoc test where appropriate. Two-Way-ANOVA was chosen because the data was found to be normally distributed and the variances were equal. ANOVA is a powerful test to analyse differences between more than two treatment groups, and Two-Way ANOVA can be used when the effect of two independent variables is to be explored (in this case dose and surface functionalisation).

2.13 Assessing Cytotoxicity After cat-MF Labelling

Theory

The outer cell membrane is a vital structure because it maintains a particular intracellular environment, and shields it from uncontrolled entrance of external fluids, molecules and organisms. Disruption of the cell membrane impacts on normal cell functions and often leads to cell death. Therefore, assessing membrane integrity after nanoparticle exposure gives an indication of potential cytotoxic effects that are mediated through disruption of the cell membrane. One way of probing if the cell membrane is still intact, is the detection of lactate dehydrogenase (LDH) in the cell culture medium. LDH is normally confined to the intracellular space and is released into the surrounding medium if the cell membrane is disrupted. Extracellular LDH can be detected in the medium using a colourimetric assay through a coupled enzymatic reaction in which LDH catalyses the conversion of lactate to pyruvate via NAD^+ reduction to NADH. The enzyme diaphorase is also added, which uses NADH to reduce the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium (INT) to a red formazan product that can be measured spectrophotometrically at 490 nm (Fig. 2.25) [59]. The amount of formazan is directly proportional to the amount of LDH released into the medium, and thus indicative of cytotoxicity.

The assay is sensitive to the number of cells, as well as to the presence of serum in the culture medium, which can give false positives. The manufacturer recommends the use of no more than 5% (v/v) foetal bovine serum (FBS) in the culture medium.

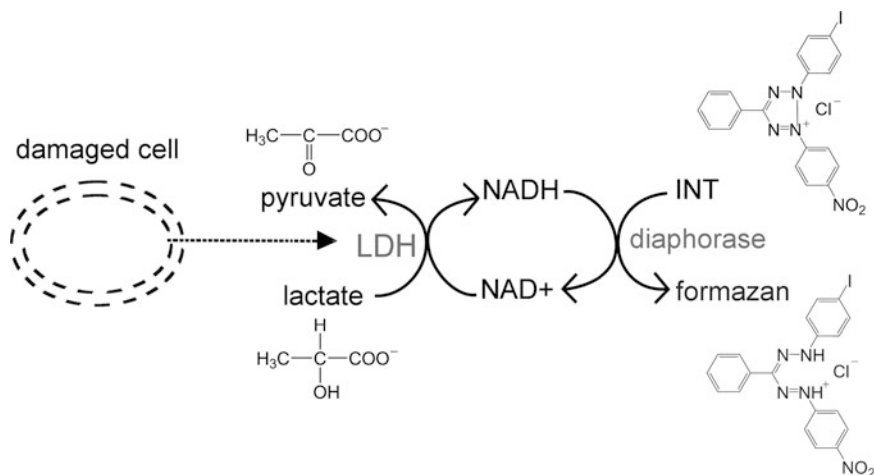


Fig. 2.25 Chemistry underlying the LDH assay. Damaged cell membrane leads to the leakage of LDH. Extracellular LDH is detected through a coupled enzymatic reaction in which LDH catalyses the conversion of lactate to pyruvate via NAD^+ reduction to NADH . Diaphorase uses NADH to reduce a tetrazolium salt (INT) to a red formazan product

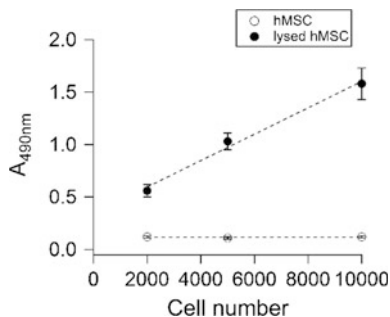
2.13.1 Optimisation of the LDH Assay

Formation of the coloured product that is measured in the LDH assay is highly dependent on the number of cells present in the sample. Therefore, an appropriate number of cells needs to be chosen to ensure the validity of the assay. The ideal cell number can be determined by performing the assays on a variety of cell numbers and determining which cell numbers produce a signal that lies within the linear range of the assay. Because the signal is generated from intracellular LDH leaked into the culture medium, cells membranes were disrupted using a surfactant-based solution (lysis buffer) to achieve maximum LDH release.

1000 to 10000 hMSCs were seeded into wells of a 96 well plate in triplicate, and incubated over night in 100 μL culture medium containing 5% of FBS, and culture medium containing 5% of FBS and 10% (v/v) of a lysis buffer supplied by the manufacturer (LDH Cytotoxicity Assay Kit, Pierce). 50 μL samples of the medium supernatant were transferred to a separate 96 well plate, where they were mixed with an equal volume of "reaction mixture" supplied by the manufacturer (although the exact composition of this mixture is not disclosed by the supplier, it likely contains all the substrates needed for LDH detection, namely lactate, NAD, INT and diaphorase). After a 30 min incubation period at room temperature, 50 μL of a "stop solution" was added. Formation of the red formazan product was assessed by measuring absorbance at 490 nm using a spectrophotometer (SpectraMax M5e, Molecular Devices).

Incubation of the LDH reagent with cell culture medium from lysed hMSCs showed a linear increase in the absorbance at 490 nm over the whole range of cell

Fig. 2.26 Absorbance in cell culture supernatants of 2000 to 10000 untreated or lysed hMSCs incubated for 30 min with LDH reagent at room temperature



numbers ($r^2 = 0.99$, Fig. 2.26). Medium supernatants taken from untreated hMSCs and incubated with the LDH reagent did not result in an increase in absorbance at 490 nm, indicating that LDH is only released upon disruption of the cell membrane.

2.13.2 LDH Assay After Exposure to MF and cat-MF

For the assessment of cytotoxicity after cat-MF exposure using the LDH assay, 5000 cells from three different patients were seeded in DMEM medium containing 5% FBS in quadruplicate into wells of a 96 well plate, left to adhere overnight and exposed for 30 min to 125 μ L of MF or cat-MF at concentrations ranging from 0.01 to 3 μ M, PBS only (untreated control), or a Lysis Buffer provided by the manufacturer as a positive control. The supernatant was removed, cells washed with PBS and cultured in DMEM with reduced serum content (5% FBS) for 24 h after exposure to native MF or cat-MF, because higher serum concentrations are known to interfere with the assay. The assay was performed using the cell culture supernatants as described for the optimisation experiment above.

Percentage cytotoxicity was calculated as follows:

$$\%_{\text{cytotoxicity}} = \frac{LDH_{MF} - LDH_{sp}}{LDH_{\max} - LDH_{sp}} * 100 \quad (2.17)$$

where LDH_{MF} was measured in cells exposed to MF/cat-MF, the spontaneous LDH, LDH_{sp} , was measured in the untreated cell control and maximum LDH, $LDH_{\max}$, was measured in cells treated with Lysis Buffer.

For statistical analysis, data were presented as mean $\pm$ standard deviation of three biological repeats (total $n = 12$). Statistical analysis was performed using IBM SPSS Statistics version 21. As for the MTS and Alamar Blue assay, the means of groups treated with MF, cat-MF, and PBS (untreated control) were compared using Two-Way ANOVA, in which effects of dose as well as surface functionalisation (unfunctionalised vs. cationised) were investigated (see Sect. 2.12.2 for rationale behind statistical analysis).

2.14 Long-Term Assessment of Cell Proliferation

Proliferation of hMSCs was assessed over a three week period using a cell counting assay. 150000 cells from three different patients were seeded in culture medium into 75 cm² tissue culture flasks and left to adhere overnight. hMSCs were exposed for 30 min to 3 mL of 0.5 μ M cat-MF or PBS (untreated cell control). The supernatant was removed, cells washed with PBS and cultured in DMEM for up to three weeks. At weekly intervals, cells were harvested, counted, and population doublings, *PD*, were calculated as follows:

$$PD = \frac{\log\left(\frac{n}{150,000}\right)}{\log 2} \quad (2.18)$$

where n was the number of counted cells, and 150000 denotes the number of cells initially seeded. Population doublings of the cat-MF treated cells were normalised to the untreated cell control. The population doubling values of cat-MF labelled and untreated hMSCs were compared at each interval using the Wilcoxon Signed-Rank test in IBM SPSS Statistics version 21. A non-parametric test was chosen because of the small sample size ($n = 3$), which made it difficult to confirm normality for the data sets. The Wilcoxon Signed-Rank test was used because two related samples were compared at each interval (i.e. week one, two and three): the untreated cells derived from three patients *versus* the cat-MF-treated cells derived from the same three patients.

2.15 Adipogenesis of hMSCs Exposed to cat-MF

Theory

Differentiation potential of hMSCs can be assessed by growing cells in a monolayer culture, while supplementing the culture medium with adipogenic growth factors, such as 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin [60]. Adipogenesis can be confirmed visually by staining lipid-rich vacuoles within the cells with a fat-soluble dye. Oil Red is a dye that functions as an oil-soluble stain. It is prepared as a solution in isopropanol and partitions into lipid vacuoles upon incubation with adipocytes, because of its increased solubility in fatty substances compared to isopropanol [61].

Method

74000 hMSCs from one patient were seeded in culture medium into the inner wells of a 24 well plate and left to adhere overnight. The outer wells were filled with **antifungal water**, containing 0.1 mg mL⁻¹ penicillin and streptomycin, and 2.5 μ g mL⁻¹ amphotericin B in sterile dH₂O. Cells were exposed to 0.75 mL of 1 μ M of cat-MF or PBS (untreated control) for 30 min. The supernatant was removed, cells washed in PBS and cultured in α MED medium with 10% (v/v) FBS,

1% (v/v) penicillin/streptomycin and 1% (v/v) glutamax solution, containing $10\ \mu\text{L mL}^{-1}$ human adipogenic supplement (StemXVivo, R&D Systems) for three weeks with media changes performed twice a week. After three weeks, adipocyte formation was assessed using Oil Red staining of intracellular lipid vacuoles. Medium was removed, cells were washed with PBS and fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature. The fixative was removed and the cells washed first with PBS followed by 60% (v/v) isopropanol. Oil Red dye was prepared by stirring $5\ \text{mg mL}^{-1}$ of Oil Red in isopropanol overnight. The dye was diluted to $3\ \text{mg mL}^{-1}$ using dH_2O and then passed through filter paper to remove aggregates. Cells were incubated with 0.5 mL of Oil Red stain for 30 min at room temperature. The stain was aspirated and cells washed with 60% (v/v) isopropanol. Stained monolayers were observed using an inverted light microscope at 10 X magnification (Leica DM IRB).

2.16 Osteogenesis of hMSCs Exposed to cat-MF

Theory

Analogous to adipogenic differentiation, the osteogenic differentiation potential of hMSCs can be assessed by growing cells in a monolayer culture, while supplementing the culture medium with osteogenic growth factors, e.g. dexamethasone, β -glycerol phosphate and ascorbate [60]. Osteogenesis can be confirmed visually by staining calcium deposits. Undifferentiated hMSCs have no extracellular calcium deposits, whereas differentiated osteoblasts produce vast extracellular calcium deposits [60]. Alizarin Red is a dye that forms a complex with calcium ions, resulting in a red product [62].

Method

37000 hMSCs from one patient were seeded in culture medium into the inner wells of a 24 well plate and left to adhere overnight. The remaining outer wells were filled with antifungal water. Cells were exposed to 0.75 mL of $1\ \mu\text{M}$ of cat-MF or PBS (untreated control) for 30 min. The supernatant was removed, cells washed in PBS and cultured in αMEM medium with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin and 1% (v/v) glutamax solution, containing $50\ \mu\text{g mL}^{-1}$ human osteogenic supplement (StemXVivo, R&D Systems) for three weeks with media changes performed twice a week.

Osteoblast formation was visualised using Alizarin Red staining of calcium phosphate deposits. Cells were washed with PBS and fixed for 1 h at $4\ ^\circ\text{C}$ using 0.5 mL ice-cold 70% (v/v) ethanol. Alizarin Red dye was prepared by stirring $13.75\ \text{mg mL}^{-1}$ of Alizarin Red in dH_2O overnight. The pH was adjusted to 4.1 using potassium hydroxide and the dye was passed through filter paper to remove aggregates. The fixative was removed and the cells were incubated with 0.5 mL alizarin red solution for five minutes at room temperature. The cells were then washed five times with PBS.

2.17 Chondrogenesis of hMSCs Exposed to cat-MF

Theory

Unlike adipogenesis and osteogenesis, chondrogenic differentiation capacity of hMSCs is often investigated in a three dimensional culture, because chondrocytes lose their typical characteristics in monolayer culture [63]. Here, hMSCs were seeded onto fibronectin-coated PGA scaffolds and treated with chondrogenic growth factors such as transforming growth factor- β 3 (TGF- β 3) [60]. PGA has proven to be one of the most suitable scaffold materials for cartilage growth, [64] and is mostly used in the form of an unwoven fiber mesh (Fig. 2.27).

Method

1000000 hMSCs were seeded in culture medium into 75 cm² tissue culture flasks and left to adhere overnight. The cells were exposed to 3 mL of 0.5 μ M of cat-MF or PBS for 30 min. The cat-MF or PBS supernatant was removed, cells were washed with PBS and left in culture medium for 24 h. After that, hMSCs were harvested with trypsin/EDTA and counted. 300000 cells were suspended in 30 μ L of culture medium and loaded onto fibronectin-coated PGA tissue engineering scaffolds of 5 mm diameter and 2 mm thickness and placed in agarose coated wells of a 24 well plate. Cells were left to attach to the scaffold over night, and then cultured for one week in DMEM containing 4500 mg glucose/L, 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamax, 1% (v/v) sodium pyruvate, and 1% (v/v) insulin-transferrin-sodium selenite, supplemented with 100 nM dexamethasone, 80 nM ascorbic acid and 10 ng/ml TGF- β 3. Three media changes were performed during this first week. After that, the medium was additionally supplemented with 10 ng/ml insulin and scaffolds were cultured for a further four weeks

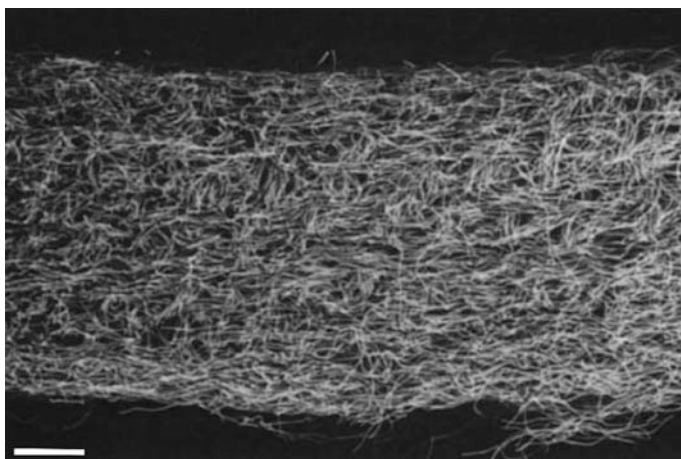


Fig. 2.27 Scanning electron micrograph of a PGA scaffold cross section. Scale bar 1 mm. Image re-printed with permission from Freed et al. [65]

with media changes three times a week. After five weeks in total, half of the scaffolds were stored at -80°C until biochemical analysis, and the other half was fixed in 4% (w/v) paraformaldehyde over night at room temperature, dehydrated in 70% ethanol for 2 h and then submitted to the histology service of the School for Cellular and Molecular Medicine at the University of Bristol. Histological sections of 5 μm thickness were stained with H&E (see Sect. 2.8.2) and Safranin O (see Sect. 2.8.3). They were also stained for iron deposits using Prussian Blue (see Sect. 2.8.4), and for collagen type II using immunostaining (see Sect. 2.8.5). Stained cartilage sections were observed using an inverted light microscope at various magnifications (Leica DM IRB).

The engineered cartilage constructs were also digested for biochemical analysis of type II collagen and glycosaminoglycans (GAG) (see Sects. 2.9.2 and 2.9.3)

2.18 Bacterial Culture

2.18.1 Liquid Culture

T7 Express BL21 carbenicillin-resistant *Escherichia coli* (*E. coli*) bacteria were kindly provided by Dr. James Armstrong (School of Cellular and Molecular Medicine, University of Bristol). 10 mL of sterilised Lysogeny Broth (LB) medium, containing 10 g L^{-1} bacto-tryptone (BD Biosciences, USA), 5 g L^{-1} bacto-yeast extract (BD Biosciences, USA) and 10 g L^{-1} of sodium chloride in dH_2O , and supplemented with 10 μL of a 50 $\mu\text{g mL}^{-1}$ carbenicillin solution (Apollo Scientific, UK) was inoculated with a sterile pipette tip dipped into *E. coli* glycerol stocks. This starter culture of bacteria was incubated over night in a rotary shaker at 200 rotations per minute (rpm) and 37°C and used the next day for experiments. Fresh starter cultures were prepared from glycerol stocks prior to each experiment.

Staphylococcus aureus (*S. aureus*) SH1000 (chloramphenicol resistant) were a kind gift from Ramesh Wigneshweraraj and Andrew Edwards, Imperial College London. Liquid cultures were prepared as described for *E. coli*, but using Nutrient broth (1 g L glucose, 15 g L peptone, 6 g L sodium chloride and 3 g L yeast extract), supplemented with 3.4 $\mu\text{g mL}^{-1}$ chloramphenicol.

2.18.2 Glycerol Stocks

Glycerol stocks were prepared by centrifuging 1 mL of bacterial suspension at $12300 \times g$ for five minutes. Half of the broth supernatant was replaced with glycerol and the suspension was vortex mixed and kept at -80°C .

2.18.3 Quantification of Bacteria in Water Samples

Theory

In this work, two methods for the quantification of bacteria were explored: measurement of the absorbance of bacterial suspensions using UV/Visible spectroscopy, and spreading samples onto nutrient rich agar plates followed by quantification of the number of colony forming units (cfu) that have grown after 24 h incubation at 37° C.

The plate count method is an indirect measure of cell density and reveals information on the number of live bacteria, whereas measuring the absorbance yields information on the amount of all bacteria in the suspension, dead and alive. For the plate count method a dilution series is prepared such that the bacteria plated out are dilute enough to be counted accurately (between 20 and 200 colonies should be present). It is then assumed that in a dilute bacterial suspension each viable cell is separate from the others and will develop into a discrete colony, such that the number of colonies reflects the number of bacteria that were present in the sample [66]. The *E. coli* strain used in the present experiments is resistant against the antibiotic carbenicillin, and by adding this antibiotic to the nutrient agar it was ensured that only *E. coli* and no other bacteria could grow on the plate.

Increasing absorbance in a bacterial culture is an indicator for bacterial growth. The amount of light scattered by a suspension of bacteria increases as the cell population increases, such that the absorbance indirectly reflects the number of bacteria present (see Sect. 2.2.2). This method is faster than the plate count method, but its sensitivity must be evaluated using a range of bacteria concentrations.

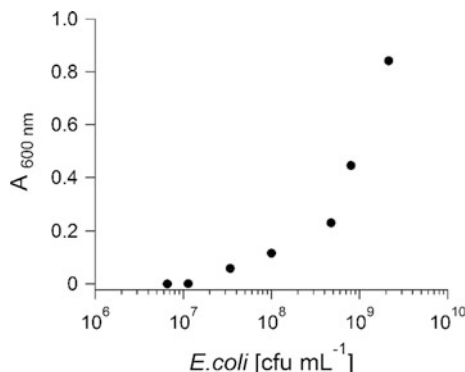
Method

UV/visible spectroscopy of liquid bacterial cultures using absorbance measurements at $\lambda = 600$ nm is described in Sect. 2.2.2.

For the plate counting method, plates were prepared in proximity to the flame of a Bunsen burner to maintain sterile conditions. Approximately 15 mL of molten 1.6% (w/v) LB agar, supplemented with 50 ng mL⁻¹ carbenicillin, were poured into sterile plastic petri dishes. The agar was set at 4 °C for at least 1 h. A range of *E. coli* suspensions were prepared and their absorbance measured as described in Sect. 2.2.2. These suspensions were further diluted in a series of 1 in 10, and 0.1 mL of the lowest three concentrations were plated out in triplicate using sterile, L-shaped plastic spreaders (VWR, UK). The plates were incubated overnight at 37 °C and cfu counted on the plates that contained 20 to 200 colonies. Bacterial concentration in cfu mL⁻¹ was determined by taking the dilution factor into consideration.

E. coli concentration could be related to a measurable absorbance values down to approximately 10⁷ cfu mL⁻¹ (Fig. 2.28). Below this value absorbance readings were not sensitive enough to accurately determine the number of *E. coli* present, because of insufficient scattering from the dilute cell suspension. Therefore, the plating method was chosen to quantify *E. coli* in the subsequent decontamination experiments. However, absorbance measurements were used to adjust bacterial

Fig. 2.28 Absorbance measured at 600 nm as a function of *E. coli* present in the water sample as determined by the plate count method. Below 10^7 cfu mL⁻¹ absorbance readings are not sensitive enough to accurately determine cell number



concentration in inoculated water samples prior to magnetic separation to approximately 10^8 cfu mL⁻¹, which corresponded to an absorbance value of around 0.3. Assessing absorbance of a bacterial suspension was first introduced in 1907 [67] and has since widely been used to achieve suspensions with a specific concentration of bacteria [68].

2.19 Magnetic Labelling of *E. coli*

All magnetisation experiments were conducted in proximity to the flame of a Bunsen burner to keep sterile conditions. The plate counting method (see Sect. 2.18.3) was used throughout to determine bacterial concentration before and after MACS.

2.19.1 Rapidity and Extent of *E. coli* Magnetisation

A 1 mL sample of liquid starter culture was centrifuged for 5 min at $14000\times g$, the medium supernatant removed, and the pellet re-suspended in 1 mL of sterile dH₂O. This suspension was diluted with sterile dH₂O to achieve a bacterial suspension with an absorbance of approximately 0.3. 0.3 mL of this suspension was mixed with an equal volume of sterile dH₂O or 1 μ M of cat-MF (sterile filtered) to yield a final cat-MF concentration of 0.5 μ M. The mixture was agitated briefly, and then incubated at room temperature for 1, 5, 15 and 30 min. After each incubation period, 0.5 mL of the bacterial suspension was loaded onto MACS columns and the flow-through was collected.

To determine cfu mL⁻¹ in the bacterial suspension before MACS, samples of the initial bacterial suspension were diluted $1/10^5$, $1/10^6$ and $1/10^7$. 0.1 mL samples of these dilutions were plated out in triplicate and incubated at 37 °C overnight. To

determine the number of cfu in the water after MACS, samples of the flow-through were diluted 1/10 to 1/10², plated out in triplicate and incubated at 37 °C overnight. After incubation, the number of colonies on the agar plates was counted and cfu mL⁻¹ determined by taking into account the respective dilution factors.

The **percentage removal** was calculated as follows:

$$removal(\%) = 100 - \left(\frac{c_{after}}{c_{before}} * 100 \right) \quad (2.19)$$

where c_{after} was the bacterial concentration in the flow-through after MACS, and c_{before} was the bacterial concentration in the initial bacterial suspension before MACS.

2.19.2 *Magnetisation of E. coli with Different cat-MF Concentrations*

A 1 mL sample of liquid starter culture was centrifuged for 5 min at 14000×g, the medium supernatant removed, and the pellet re-suspended in 1 mL of sterile dH₂O. This suspension was diluted in sterile dH₂O to achieve a bacterial suspension with an absorbance of approximately 0.3. 0.3 mL of this suspension was mixed with an equal volume of sterile water or various concentrations of cat-MF (sterile filtered) to yield a final cat-MF concentration of 0.01 to 1 µM cat-MF. The mixture was agitated briefly, and then incubated at room temperature for 15 min. After the incubation period, 0.5 mL of the bacterial suspension was loaded onto MACS columns, the flow-through was collected and cfu mL⁻¹ and percentage removal determined as described in Sect. 2.19.1.

2.19.3 *Magnetic Capture of Various Concentrations of E. coli*

A 1 mL sample of liquid starter culture was centrifuged for 5 min at 14000 × g, the medium supernatant removed, and the pellet re-suspended in 1 mL of sterile dH₂O. The suspension was diluted in sterile dH₂O to achieve a bacterial suspension with an absorbance of approximately 0.3. This suspension was further diluted in a series of 1 in 10, down to a dilution of 1/10⁶. 0.3 mL of each dilution was incubated with an equal volume of 1 µM of cat-MF (sterile filtered) to yield a final cat-MF concentration of 0.5 µM. The mixture was agitated briefly, and then incubated at room temperature for 15 min. 0.5 mL of the bacterial suspension were loaded onto MACS columns, the flow-through was collected and cfu mL⁻¹ and percentage removal determined as described in Sect. 2.19.1.

2.19.4 Concentration of Low Amounts of *E. coli*

A 1 mL sample of liquid starter culture was centrifuged for 5 min at $14,000\times g$, the medium supernatant removed, and the pellet re-suspended in 1 mL of sterile dH_2O . This suspension was diluted in sterile dH_2O to achieve a bacterial suspension with an absorbance of approximately 0.3. This suspension was further diluted in a series of 1 in 10, down to a dilution of $1/10^7$. 0.3 mL of this dilution was incubated with an equal volume of 1 μM of cat-MF (sterile filtered) to yield a final cat-MF concentration of 0.5 μM . The mixture was agitated briefly, and then incubated at room temperature for 15 min. 2 mL of the bacterial suspension were loaded onto MACS columns, the flow-through was collected. The column was removed from the magnet and any bacteria immobilised in the column were eluted with 0.4 mL of sterile dH_2O using a plunger. The initial solution, the flow through and the eluted bacterial suspension were plated out without further dilution. cfu mL^{-1} and percentage removal were determined as described in Sect. 2.19.1.

2.20 Comparing Magnetic Capture of *E. coli* and *S. aureus*

1 mL samples of liquid starter culture of *E. coli* and *S. aureus* were centrifuged for 5 min at $14000 \times g$, the medium supernatant removed, and the pellets re-suspended in 1 mL of sterile dH_2O . The suspensions were diluted in sterile dH_2O to achieve a bacterial suspension with an absorbance of approximately 0.3. 0.3 mL of each suspension was mixed with an equal volume of sterile dH_2O or 1 μM of cat-MF (sterile filtered) to yield a final cat-MF concentration of 0.5 μM . The mixtures were agitated briefly, and then incubated at room temperature for 15 min. 0.5 mL of each bacterial suspension were loaded onto MACS columns, the flow-through collected and cfu mL^{-1} and percentage removal determined as described in Sect. 2.19.1. Note that *S. aureus* were plated out on Nutrient agar rather than LB agar.

2.21 Assessing *E. coli* Growth in the Presence of cat-MF

A 0.5 mL sample of liquid starter culture was diluted in LB broth medium containing 50 ng mL^{-1} carbenicillin to achieve a bacterial suspension with an absorbance of approximately 0.1. 10 μL of this *E. coli* suspension was added to 180 μL of carbenicillin supplemented LB broth containing 0.01 to 1 μM cat-MF. The absorbance of the bacterial solution, as well as of the background solutions, was

recorded every 10 min at over 4 h at 37 °C. The absorbance of the background solutions were subtracted from the bacterial suspensions to account for any absorbance effects due to the presence of cat-MF particles.

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