Electronic Supplementary Material (ESI) for Soft Matter.

Supplemental information

Effect of metal ions on physical properties of multilayers from hyaluronan and chitosan and adhesion and growth of multipotent mouse fibroblasts

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1. Ellipsometry

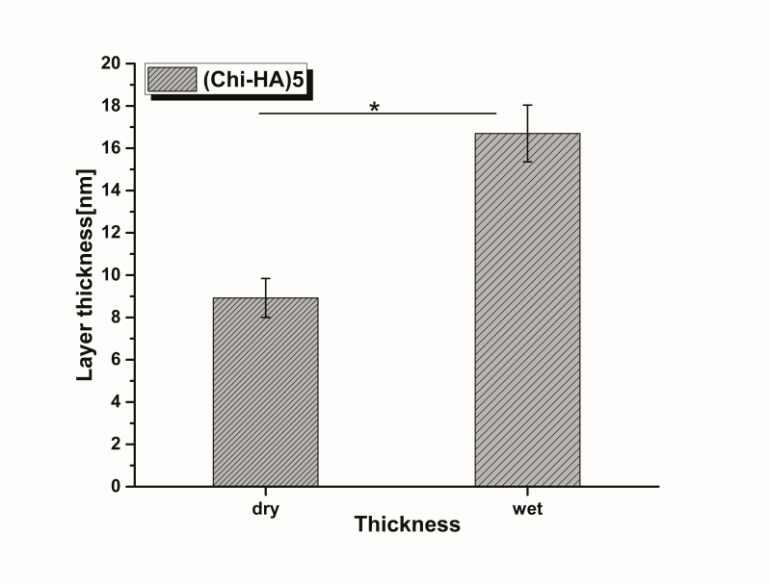

Figure S1: Calculated dry and wet thicknesses of $[Chi/HA]_5$ multilayers studied by ellipsometry. Results represent means ± SD.

2. Freestanding film formation

The LbL method was used to fabricate $[CHI/HA]_{100}$ freestanding multilayer films. Films containing 100 bilayers of (2 mg/ mL) Chitosan (Chi) and (5 mg/ mL) Hyaluronic acid (HA) were fabricated using an automated dip coating device (DR01, Riegler & Kirstein, Berlin, Germany). The freestanding films Chi/HA were assembled on poly (propylene) (76 X 26mm2, Halle, Germany) to permit the fabrication and detachment of films. The dip-coating process was performed as reported previously [1]. For cross-linking with metal ions, the films were detached from the substratum and cut into circular disks of 12 mm diameter and placed inside 24-well plates. Then the films were incubated with metal ions solutions ($Cu^{2+,}$ Co^{2+}, Ca^{2+}, and Fe^{3+}) of highest concentration for 30 min incubation followed by three times rinsing with 0.15 M (NaCl) for 5 min each. (**Figure S2)**

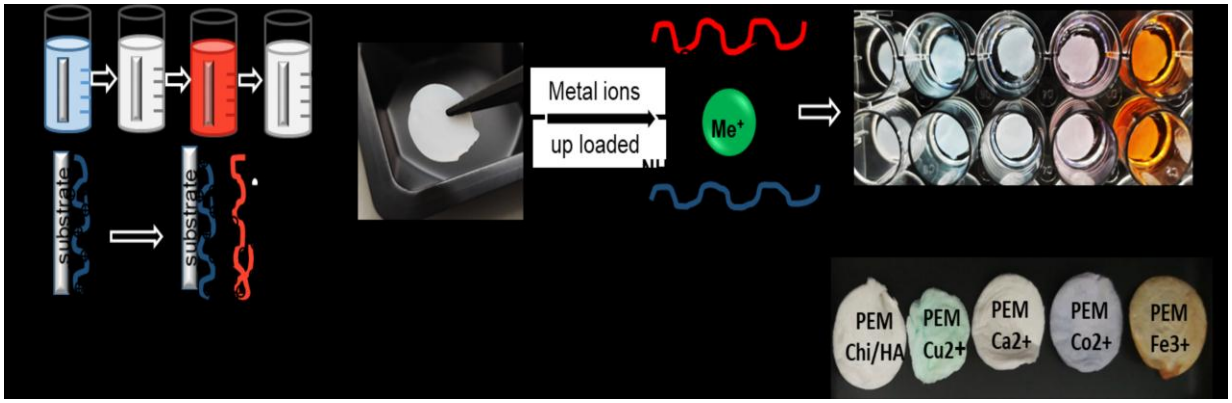

Figure S2: Scheme of freestanding multilayer film preparation with subsequent crosslinking with high concentrations of metal ions and photographs of the resulting films with obvious staining by the metal ions.

3. FTIR spectroscopy

Figure S3 shows that the spectrum of pure Chi presents a broad absorbance band at about 3275 cm^{-1} related to the corresponding amine N–H and hydroxyl group O–H, including those from residual water. Furthermore two bands at 2980 and 2881 cm^{-1} caused by stretching of C-H; the absorption band of amide I stretching at 1651 cm^{-1}, and bending vibrations of the N-H (N-acetylated residues, amide II band) at 1587 cm^{-1} were found [2]. Amine deformation vibrations usually produce strong bands in the range of 1638-1575 cm^{-1}. Hence, the peak at 1587 cm^{-1} can be also a contribution of the N-H bending of the amine, as previously discussed [3]. The peaks at 1419 and 1377 cm^{-1} belong to the deformation of C-H and the stretching of C-N, respectively [4-5]. The absorption bands at 1150 cm^{-1} (anti-symmetric stretching of the C-O-C bridge and C-N stretch), 1075 cm^{-1}, 1050 cm^{-1} and 1030 cm^{-1} (skeletal vibrations involving the C-O stretching) are characteristics of its saccharide structure[2, 3].

The spectrum of HA (see **Figure S3** as well) shows an intense band that has its maximum at about 3275 cm^{-1} attributed to N-H and O-H groups engaged in hydrogen bond formation and some residual water after drying of free-standing films. The band at around 2900 cm^{-1} can be referred to stretching vibration of the C-H bonds. The carbonyl band $\nu_{C=O}$ of the (protonated) carboxylic group COOH appears at 1730 cm^{-1}; this group has also been assigned to the peak at 1608 cm^{-1} [6] . This zone is where amide I and amide II are expected

and probably their contributions superpose, the peak at 1555 cm^{-1} attributed to the amide II vibration [7]. The bands at about 1400 cm^{-1} are also characteristic of hyaluronic acid and correspond to C=O and C-O bonds in the carboxylate[8]. The intense band extending between 1200 and 900 cm^{-1} corresponds to the saccharide unit C-O-C stretching vibration (1150 cm^{-1} O-bridge, 1070 cm^{-1} and 1024 cm^{-1} C-O vibration) [9].

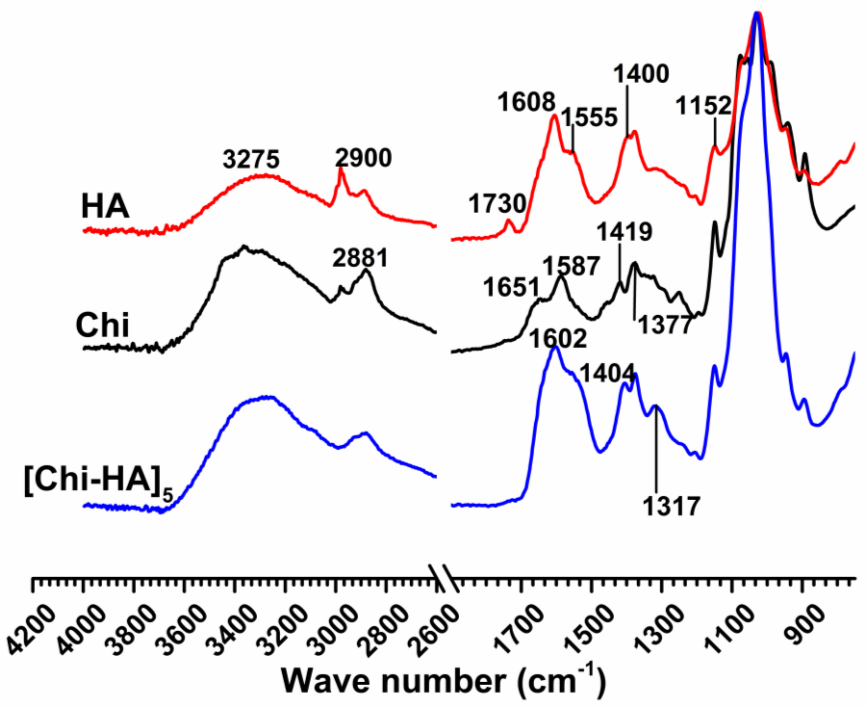

Figure S3: FTIR spectra of pure chitosan (Chi), hyaluronic acid (HA) and dry $[Chi/HA]_{100}$ multilayer films

4. Cytotoxicity studies

For cytotoxicity studies, cell were seeded in 96 well plates at a density of 5×10^4 cells/mL in EBM supplemented with 10% FBS and 1% pen/strep and incubated at 37 °C in a humidified 5% CO_2/95% air atmosphere with different (concentration and type) metal ions for 24 h and 72 h. The metabolic activity of C3 H10T1/2 cells was determined using the non-toxic QBlue® assay (BioChain, USA). Therefore, the old medium was carefully aspirated and 200 µL of pre-warmed EBM containing the QBlue reagent (ratio 1:10) was added to each well. The samples were again incubated at 37 °C for 3 h, and then 100 µL of supernatant from each well was transferred to a 96-well black plate (Greiner). The fluorescence intensities were

measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm with a fluorescence plate reader (BMGLABTECH, Fluostar OPTIMA, Offenburg, Germany).

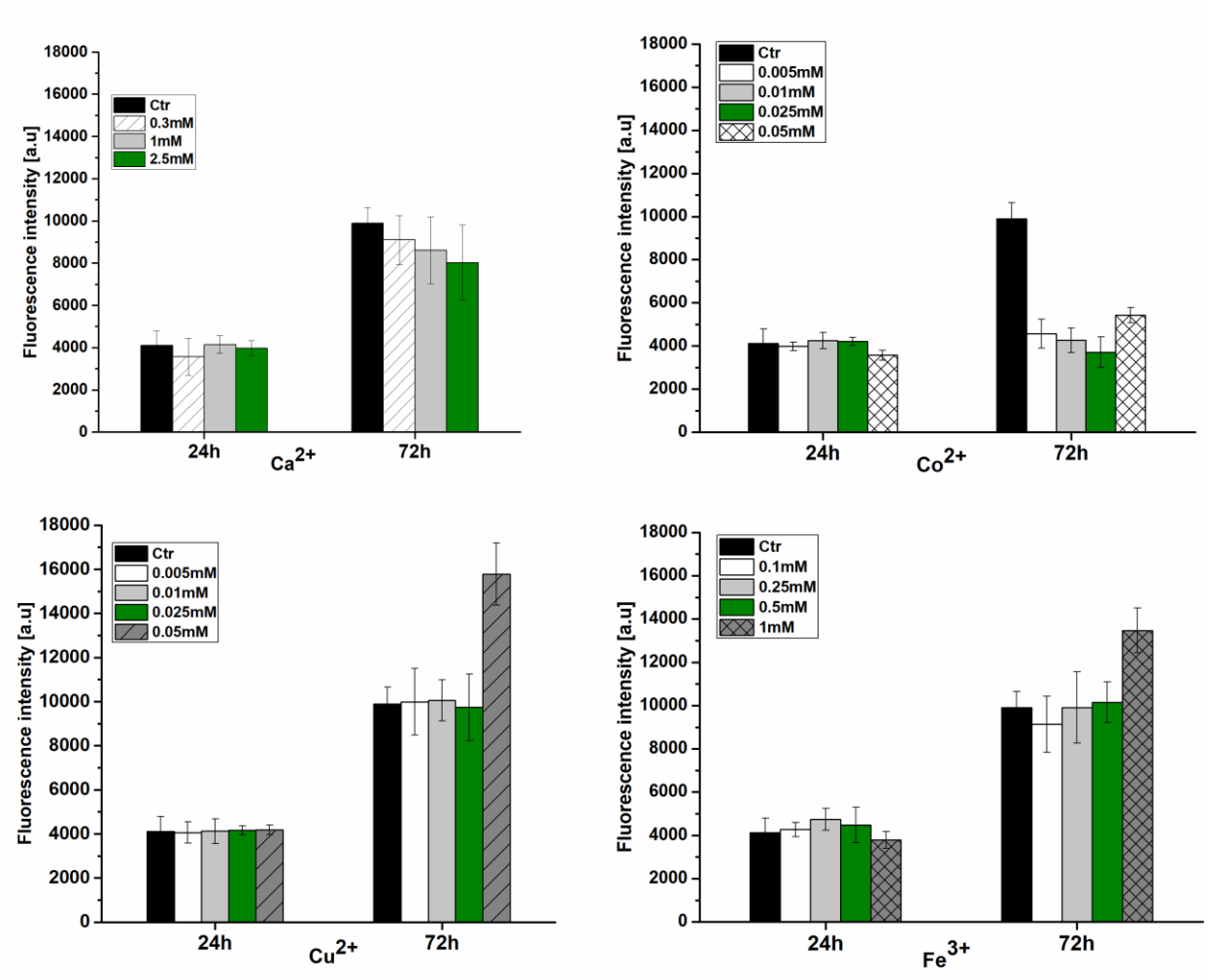

Figure S4: C3H10T1/2 cells seeded on 96 well plate for 24h. The cells were incubated with EBSM medium with 10%FBS and addition of different metal ions (type and concentration). Cell viablity was determined by QBlue assay after 24 and 72 h of culture. Results are means ± SD.

To clarify whether a reduced quantity of cells on multilayers cross-linked with metal ions is due to toxic effects, . cell viability studies were performed by pre-culturing C3H10T1/2 cells on 96 well plates and exposing them to solutions of the chloride salts of the metal ions in a cell culture medium supplemented with 10% FBS for 24 and 72 has shown in **Figure S 4**. It was found that micro molar concentrations of Co^{2+} ions (5 µM) had no cytotoxic effect after

24 h, but inhibited further proliferation of cells in this concentration range up to 50 µM. By contrast, Cu^{2+} did not show any cytotoxicity and growth inhibition in comparison to the control at the same concentration range. In addition, calcium and iron ions that were applied in the 2.5 mM range did not show any signs of cytotoxicity or growth-inhibiting effects on C3H10T1/2 cells. However, significant effect on cell growth was seen for Co^{2+} which seems to inhibit cell growth during the time of incubation already at the lowed concentration of 5 µm.

Reference:

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