ABSTRACT: Layer-by-layer (LbL) polyelectrolyte coatings are intensively studied as reservoirs of bioactive proteins for modulating interactions between biomaterial surfaces and cells. Mild conditions for the incorporation of growth factors into delivery systems are required to maintain protein bioactivity. Here, we present LbL films composed of water-soluble N-[2-hydroxy-3-trimethylammonium]propyl] chitosan chloride (HTCC), heparin (Hep), and tannic acid (TA) fabricated under physiological conditions with the ability to release heparin-binding proteins. Surface plasmon resonance analysis showed that the films formed on an anchoring HTCC/TA bilayer, with TA serving as a physical crosslinker, were more stable during their assembly, leading to increased film thickness and increased protein release. X-ray reflectivity measurements confirmed intermixing of the deposited layers. Protein release also increased when the proteins were present as an integral part of the Hep layers rather than as individual protein layers. The 4-week release pattern depended on the protein type; VEGF, CXCL12, and TGF-β1 exhibited a typical high initial release, whereas FGF-2 was sustainably released over 4 weeks. Notably, the films were nontoxic, and the released proteins retained their bioactivity, as demonstrated by the intensive chemotaxis of T-lymphocytes in response to the released CXCL12. Therefore, the proposed LbL films are promising biomaterial coating candidates for stimulating cellular responses.

1. INTRODUCTION

Multicomponent coatings for the release of therapeutic agents are widely studied variants of surface modifications for functional materials in the biomedical field. The deposition of ultrathin films on the surface of biomaterials can influence phenomena critical in tissue engineering (TE), such as vascularization processes, alleviation of immunological responses, and prevention of biological material adhesion. The immobilization of macromolecules, such as growth factors and chemokines, can support cell recruitment, stimulate the formation of new vasculature within implants or TE constructs, and ameliorate foreign-body reactions of the immune system, leading to efficient integration of the implant with surrounding tissues. However, the incorporation of proteins without a disruption of their complex structures while maintaining their desired biological activity is a task that requires physicochemical procedures performed under mild conditions.

Layer-by-layer (LbL) alternate deposition of oppositely charged polyelectrolytes is a technique used to obtain nanoscale coatings on implant surfaces with tunable properties such as antibacterial activity, ability to stimulate or inhibit protein and cell adhesion, and the ability to release bioactive agents. To date, several types of LbL systems for the delivery of growth factors have been reported and are reviewed in refs 3, 7, 8. Among the polyelectrolytes of synthetic and natural origin assembled in protein-delivering LbL films, polysaccharides have recently gained attention due to their biocompatibility, bioactivity, and easy functionalization, making them perfect candidates to play the role of polyelectrolytes. Negatively charged glycosaminoglycans, such as dextran sulfate, hyaluronic acid (HA), chondroitin sulfate, and heparin (Hep), are often used as polyanionic components. Among them, heparin may be beneficial in terms of the effective adsorption of so-called heparin-binding proteins due to their natural affinity for this polysaccharide. Therefore, heparin-binding proteins, such as growth factors (e.g., fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF)), cytokines (e.g., transforming growth factor-β (TGF-β1)), and chemokines (e.g., stromal cell-derived factor...
2. EXPERIMENTAL SECTION

2.1. Materials. Chitosan (LMW, degree of deacetylation (DDA) 77.6%), heparin sodium salt from porcine intestinal mucosa (Hep), tannic acid (TA), glycylidtrimethylammonium chloride (GTMAC, ≥90%), 2-mercaptopethanesulfonic acid (MESNA), bovine serum albumin (BSA), polyethyleneimine (branched, 25 kDa), lysozyme (specific activity ≥70,000 units/mg protein), phosphate-buffered saline (PBS), sodium azide, acetic acid (CH₃COOH), UV-

If proteins are incorporated into a film structure as individual building blocks or as a part of a cargo that can also serve as a film component, we assume that the amount of protein in the coating increases. Higher protein loading can facilitate the achievement of biologically effective doses of the protein released. In the present work, we studied the self-assembly of LbL films consisting of HTCC, Hep, and heparin-binding proteins and the ability of HTCC/Hep LbL films to deliver CXCL12, FGF-2, VEGF, and TGF-β1. To investigate how the film structure affects protein release, the proteins were loaded into the assembly as (i) single individual layers or (ii) a component of Hep layers, as illustrated in Figure 1. The complexation of heparin-binding protein with Hep through multilayer binding stabilizes the protein conformation. Therefore, in addition to the potential effects on protein release and shortening the assembly process, protein deposition using a protein cosolution with Hep is expected to preserve the protein bioactivity during the long-term coating process. Moreover, growth factors released from delivery systems as complexes with Hep have been found to influence the cellular response more effectively than the proteins alone. Furthermore, an anchoring bilayer consisting of HTCC and tannic acid (TA), also known to physically crosslink macromolecules at multiple binding sites through multiple interactions, including hydrogen bonding, ionic bonding, and π–π interactions, was determined to increase the film stability during the film buildup. The self-assembly of HTCC/Hep LbL films was monitored and evaluated via surface plasmon resonance, spectroscopic ellipsometry, and X-ray reflectivity measurements, and surface topography was analyzed with atomic force microscopy (AFM). The in vitro protein release was determined using an ELISA method, and the in vitro bioactivity of the released proteins was demonstrated by the migration of the human T lymphoma cell line stimulated by released CXCL12.
2.2. Synthesis of Quaternized N-[(2-Hydroxy-3-trimethylammoniumpropyl)chitosan] (HTCC). Chitosan was modified by GTMAC as described previously with minor modifications to simplify protocols frequently used in the literature. To control the reproducibility of the modification reaction, we primarily assessed the effect of the chitosan concentration and reaction temperature (i.e., 55, 65, or 75 °C) on the degree of substitution (DS). Details of the synthesis are presented in the Supporting Information, and the reaction conditions under study are summarized in Table S1. The modification was confirmed by ¹H NMR analysis (δ = 3.2 ppm corresponds to the protons of the –N(CH₃)₂ groups of GTMAC (Figure S1). The degree of substitution (DS) of HTCC was determined by a conductometric titration of the HTCC aqueous solution with the AgN₂O₃ solution (1) (for details, see the Supporting Information).

2.3. Formation of Multilayer Polyelectrolyte HTCC/Heptide Films Monitored In Situ by Surface Plasmon Resonance Spectroscopy. Self-assembly of LbL films with an HTCC/TA anchoring layer (i.e., TA-(HTCC/Heptide)₀ and TA-(HTCC/ Heptide-mix-protein)₀ (Figure 1), and the corresponding films without the anchoring layer was monitored in situ as a shift in the resonance wavelength using a custom-built surface plasmon resonance (SPR) sensor (Institute of Photonics and Electronics (IPE), Czech Academy of Sciences (CAS), IPE CAS, Prague, Czech Republic) at 25 °C. The films were deposited on gold-coated SPR chips (IPE CAS, Prague, Czech Republic) modified with MESNA (for the modification protocol, see the Supporting Information). The concentrations of the HTCC, Hep, and TA solutions were 1 mg/mL, and those of the protein solutions were 1 mg/mL. For the Hep-mix-protein mixtures, the protein was precomplexed with Hep one hour before the experiment at a ratio of Hep/protein = 25:1 w/w to give a final concentration of 26 µg/mL. The chemokey CXCL12 and Hep-mix-protein

2.4. Evaluation of Interactions between Lysozyme, Hep, and a Hep-Lysozyme Complex with HTCC in PBS. Isothermal titration calorimetry (ITC) of the solutions of a model protein lysozyme (Lz), Hep, and Hep precomplexed with Lz (Hep-Lz) at a weight ratio of 25:1) to the HTCC solution in PBS was carried out at 25 °C on a MicroCal ITC200 (Malvern Panalytical Ltd., U.K.). Global fitting of the binding isotherms was carried out with Affimint software ver. 1.2.3. (SASD - AFFINImeter, Santiago de Compostela, Spain). From the fit obtained, the affinity constant, Kᵣ (M⁻¹), stoichiometry, n (number of Hep molecules per one HTCC molecule), binding free energy (ΔG), binding enthalpy (ΔH), and binding entropy (ΔS) were calculated for 1 mol of tetratin (Hep, Hep-Lz). Details about ITC measurements are presented in the Supporting Information.

2.5. Lbl Film Assembly on Glass and Slica Substrates. 2.5.1. Substrates. Slica wafer (15 × 10 mm², 60/1-10/675±25/ SS/TTV<10, Siegent Wafer GmbH, Aachen, Germany) or round-shaped glass coverslips (with a diameter of 15 mm, Marienfeld, Germany) were activated with a freshly prepared alkaline piranha solution and subsequently by air plasma treatment (for details, see the Supporting Information). 2.5.2. Coating Process. Protein-loaded multilayer polyelectrolyte films of HTCC and Hep with individual protein layers, i.e., TA-(HTCC/Heptide/protein)₀ and combined heparin-protein layers, i.e., TA-(HTCC/Hepe-mix-protein)₀ were fabricated by the Lbl dripping approach using a custom-built computer-controlled Lbl coater (based on components and software from Standa Ltd., Vilnius, Lithuania). The plastic vials used for the protein-containing solutions were precoated with a 1 wt % solution of BSA in PBS. All polyelectrolyte solutions were prepared in PBS (pH 7.4) and filtered under sterile conditions with 0.22 µm pore size filters. The concentrations of the HTCC, Hep, and TA solutions were 1 mg/mL, and those of the pure protein (i.e., CXCL12, VEGF, and Hep-2) solutions were 1 µg/mL. For the Hep-mix-protein mixtures, the protein was precomplexed with Hep in PBS one hour before the experiment at a ratio of Hep/protein = 25:1 (w/w) to give a final concentration of 26 µg/mL.

Film fabrication was performed under sterile conditions in a flow box, and the coating conditions were adjusted based on SPR analysis. The film construction started by immersion of the plasma-activated substrates in UV-spectroscopic-grade ethanol for 30 min, followed by deposition of the anchoring HTCC/TA bilayer (10 min for each solution). Then, the samples were alternately immersed in the solutions of HTCC (the first and second blocks for 5 min, following blocks for 3 min), Hep (10 min), and the specific protein or Hep-mix-protein solution (40 min) for preparation of the TA-(HTCC/Heptide/protein)₀, and TA-(HTCC/Hepe-mix-protein)₁ films, respectively. For comparative release studies, the anchoring PEI layer was deposited from a 5 mg/mL solution in PBS (20 min). The substrate immersion in each polyelectrolyte solution was followed by a rinsing step in PBS for 5 min. The process of sequential adsorption and rinsing was repeated to obtain the desired number of HTCC/Heptide/protein or HTCC/Hepe-mix-protein blocks.

The films assembled on silica substrates were used for spectroscopic ellipsometry, X-ray reflectivity, and AFM analyses, and the films assembled on glass substrates were used for release studies and biological evaluation experiments.

2.6. Film Characterization. 2.6.1. Spectroscopic Ellipsometry (SE). The dry thickness of the Lbl films deposited on a silica wafer and the films exposed to physiological-like conditions was measured by spectroscopic ellipsometry (M2000, J.A. Woollam Co.) working in a one-dimensional silicon strip detector Mythen 1K (Dectris, Switzerland).

2.6.2. X-ray Reflectivity (XRR). The film thickness of the first layers of the Lbl films with the HTCC/TA anchoring layer, i.e., HTCC/TA-(HTCC/Heptide)₀ was estimated from XRR measurements. XRR measurements were evaluated on multiple samples deposited on a silica wafer with a gradually increasing complexity (i.e., an increasing number of deposited layers). Reflectivity patterns were obtained using a high-resolution diffractometer Explorer (GNR Analytical Instruments, Italy) equipped with a one-dimensional silicon strip detector Mythen 1K (Dectris, Switzerland).

2.6.3. Atomic Force Microscopy (AFM). The surface topography of the Lbl films deposited on silicon wafers was evaluated with a NanoScope III Multimode Atomic Force Microscope (Veeco, Santa Barbara, CA) in tapping mode with OTESPA7 standard silicon cantilevers (Veeco Instruments), a spring constant of 42 N/m, and a scan rate of 0.6–1 Hz in air.

Details about the SE and XRR measurements are presented in the Supporting Information.

2.7. Disintegration of Lbl Films in PBS. The long-term stability of the protein-loaded HTCC/Heptide Lbl films during incubation in PBS was monitored as follows: TA-(HTCC/Heptide)₀ and TA-(HTCC/Hepe-mix-protein)₁ films prepared on silica wafers were placed in 5 mL Eppendorf vials, immersed in PBS with 0.02 wt % sodium azide (to prevent bacterial contamination), and incubated at 37 °C for 35 days. At predetermined time intervals, the samples were removed from the vials, rinsed with MQ water, dried with a N₂ stream, and subjected to SE analysis to characterize changes in film thickness.

2.8. Protein Release from Lbl Films. The TA-(HTCC/Heptide)₀ and TA-(HTCC/Hepe-mix-protein)₁ Lbl films prepared on glass coverslips were placed in 24-well cultivation plates that were...
precoated with a 1 wt % solution of BSA in PBS. The samples were incubated with 0.6 mL of PBS with 0.1% BSA and 0.02% sodium azide in a horizontal rotator under slow agitation at 37 °C. To prevent the evaporation of the release media during long-term incubation, the plates were covered with an ELISA plate sealer, closed with a plate cover, and finally sealed with Parafilm. At predetermined time intervals (1, 2, 4, 8, 24, 48, 168, 336, 504, and 672 h), the release medium was withdrawn and replaced with fresh medium. The released aliquots were kept frozen at −20 °C until the determination of the released CXCL12, FGF-2, VEGF, and TGF-β using commercial ELISA kits (for specifications, see the Supporting Information). The obtained data were recalculated for one cm² of the area of LbL films.

2.9. In Vitro Studies. 2.9.1. Cytocompatibility of LbL Films. The experiment was performed in a Milicell Hanging cell culture insert (5.0 μm pore size, Merck Ltd., Prague, Czech Republic) placed in 24-well plates (TPP, Biotech, Prague, Czech Republic). MG-63 cells (a human osteosarcoma cell line MG-63) were selected as model adherent cells. MG-63 cells were seeded onto the insert bottom at a density of 3 500 cells per insert in 100 μL of Eagle’s minimum essential medium (EMEM, Thermo Fisher, Prague, Czech Republic) specific for MG-63 cells. Next, 1 mL of EMEM was added to the bottom wells, and the cells were allowed to adhere for 24 h. Then, the medium in the wells was replaced with fresh EMEM (1 mL), TA-(HTCC/Hep), LbL films (nonloaded with protein) on glass slides were placed in each well, and the cells were cultured in 5% CO₂ at 37 °C for the next 72 h. Then, the inserts with seeded cells were placed in a new 24-well plate with fresh EMEM (1 mL) in each well, and cell viability was determined using the Resazurin assay (PrestoBlue, Figure 2. Formation of HTCC/Hep LbL films followed in situ using SPR analysis. Effect of the anchoring HTCC/TA layer (TA-) on the assembly of HTCC/Hep/CXCL12 films with the individual protein layer (A) and HTCC/Hep-mix-CXCL12 films with protein deposited as a component of the Hep layer (B). Dependence of the resonance wavelength shift on the number of deposited HTCC/Hep/CXCL12, HTCC/Hep-mix-CXCL12 and HTCC/Hep (a reference system) blocks in the films with the anchoring HTCC/TA bilayer (C). Assembly of TA-(HTCC/Hep/ protein) (D) and TA-(HTCC/Hep-mix-protein) (E) LbL films containing CXCL12, VEGF, and FGF-2. The sensogram curves in graphs are shifted vertically for better clarity. Arrows indicate injections of particular film components, and * indicates the rinsing step with PBS.
Thermo Fisher, Prague, Czech Republic) following the manufacturer’s instructions. Details about the cell cultivation and resazurin assay are presented in the Supporting Information. MG-63 cells without any treatment were used as a positive control, and cells treated with 4 mM solution of H$_2$O$_2$ in PBS were used as a negative control. The cytotoxicity evaluation was performed in three independent experiments in triplicates. Cell viability is expressed as % relative to the positive control, which was set as 100%.

2.9.2. Bioactivity of CXCL12 Released from LbL Films.

2.9.2.1. Sample Preparation. The TA-(HTCC/He$\text{p}$/CXCL12)$_{15}$ and TA-(HTCC/He$\text{p}$-mix-CXCL12)$_{15}$ LbL films prepared on glass slides were placed in 24-well cultivation plates that were precoated with a 1 wt % solution of BSA in PBS. The LbL films were incubated with 0.7 mL of RPMI cultivation medium with 1% fetal bovine serum (FBS) in 5% CO$_2$ at 37 °C for 48 h under sterile conditions. The cultivation medium with the released CXCL12 was immediately used for migration studies.

Jurkat cells (a human T lymphoma cell line) were evaluated for expression of the CD54 receptor and cell chemotaxis stimulated by soluble CXCL12 before each migration experiment. Details about the cultivation of Jurkat cells are presented in the Supporting Information. The bioactivity of CXCL12 released from the LbL films was assessed by a Boyden chamber-based cell migration assay using RPMI cultivation medium with 1% of FBS as follows: 3 × 10$^5$ Jurkat cells in 150 μL of the medium were placed into inserts (Milicell Hanging cell culture insert, 5 μm pore size, Merck Ltd., Prague, Czech Republic), which were placed in a 24-well cultivation plate (TPP, Biotech, Prague, Czech Republic). The bottom compartment of each well was filled with 650 μL of (i) the cultivation medium after sample incubation, (ii) the cultivation medium containing free CXCL12 (CXCL12 concentrations of 5 and 15 ng/mL) as a positive control, and (iii) the pure cultivation medium as a negative control. To follow the experimental history of the LbL film samples, the cultivation media used for positive and negative controls were also incubated in 5% CO$_2$ at 37 °C for 48 h prior to the migration study. The cultivation plates were incubated in 5% CO$_2$ at 37 °C for 4 h, as determined in the preliminary experiments (Figure S2). Then, the migrated cells were centrifuged, resuspended in 30 μL of PBS, and counted with a Bürker chamber. Cell migration is expressed as % relative to the negative control, which was set as 100%.

2.9.2.2. Statistical Evaluation. The results were analyzed with GraphPad Prism5 software. The statistical evaluation was conducted using one-way ANOVA with Dunnett’s multiple comparison post-test and Tukey’s multiple comparisons post-test.

3. RESULTS AND DISCUSSION

3.1. Synthesis of N-[(2-Hydroxy-3-trimethylammonium)propyl]chitosan Chloride (HTCC). The chitosan derivatives bearing quaternary ammonium side groups (Figure 1), abbreviated HTCCs, were prepared by the reaction of chitosan with GTMAC under acidic conditions. In contrast to the modification reaction in water, acidic conditions allow a homogeneous reaction with selective quaternization of the primary amine groups of chitosan, leading to better control over the modification. To control the reproducibility of the modification reaction, we assessed the effect of the polymer concentration, reaction temperature, and molar GTMAC/chitosan ratio on DS under reaction conditions frequently reported in the literature. Table S1 presents the DSs obtained for HTCCs prepared under several reaction conditions. Despite the high reaction temperature of 75 °C, which generally increases the efficiency of the substitution reaction, dilution of the reaction mixture to 180 and 250 mL led to DSs of 50 and 22%, respectively. The obtained relatively low substitution is comparable to the DSs of HTCCs prepared at similar concentrations of reactants but at only 55 °C. On the other hand, using the highest reactant concentration (i.e., 100 mL of a 0.5% eq. CH$_2$COOH solution), the increase in reaction temperature from 55 to 75 °C resulted in a significant increase in DS from 41 to 88%, even though a low GTMAC/chitosan molar ratio of 3 was used. A similar temperature dependence, although not as profound, was reported for highly concentrated chitosan solutions in an aqueous perchloric acid solution with a GTMAC/chitosan molar ratio of 8.41 Finally, DS can increase with an increasing GTMAC/chitosan molar ratio, which was also shown in our experiments when DS increased from 70 to 93% as the GTMAC/chitosan molar ratio doubled (Table S1). For the following assembly experiments, HTCC prepared at 65 °C and with a DS of 70% was used.

3.2. SPR Analysis of the Self-Assembly of HTCC and Hep. The self-assembly of HTCC, Hep, and protein was monitored in situ by SPR measurements. The structure of the HTCC/Hep LbL films differed in two main parameters: (i) the presence of the anchoring HTCC/TA bilayer (referred to as TA-) with the expected impact on film stability during the film buildup and (ii) the way the protein was incorporated into the films, i.e., as individual layers or as a part of Hep layers, as shown in Figure 1. The main study on film formation was performed on LbL films containing the protein CXCL12. The shift in a resonance wavelength (signal) after exposure of the SPR chip to polyelectrolyte solutions and PBS reflects changes in the refractive index at the interface due to either adsorption (a signal increase) or desorption (a signal decrease) of specific components on the chip surface. Comparison of the shifts in resonance wavelength gives information about the relative amount of the deposited components and assembly dynamics. SPR sensograms depicting the deposition of HTCC, Hep, and CXCL12 layers during the formation of (HTCC/Hep/CXCL12)$_n$ and (HTCC/Hep-mix-CXCL12)$_n$ LbL films (Figure 2A, red patterns) confirmed the mass deposition of the polyelectrolytes used in particular blocks. The sensogram for the reference (HTCC/Hep)$_n$ assembly without the protein is shown in Figure S3A. A slight decrease in the signal after the injection of PBS (a rinsing step, depicted as * in the sensograms) suggests a negligible difference in the refractive index between PBS and the polyelectrolyte solutions (1 mg/mL) and indicates short-term stability of the deposited films at physiological pH. However, in both types of LbL assemblies, an initial abrupt increase in the signal observed after the second and third injections of the HTCC solution was followed by a sharp signal decrease, nearly reaching the values of the previously deposited layer. Importantly, the subsequent rinsing step with PBS regularly led to immediate signal stabilization, indicating the stabilization of interactions between the deposited layers in the formed films. If the PBS rinsing step was not applied, the signal continued to decrease over time, indicating intensive desorption of the mass from the chip surface (data not shown). This observation suggests that adsorption, diffusion, and redissolution processes occur when the formed film comes into contact with the polycation solution, as was also reported for chitosan/HA, poly(dimethylaminoethyl methacrylate)/poly(acrylic acid), poly(I-lysine)/HA (PLL/HA) and HTCC/TA LbL assemblies.32,42−45 We can assume that as the LbL system tends to reach equilibrium between the formed multilayer (a form of "dense phase") and its surroundings, as proposed by Kovacevic et al.,34 the HTCC molecules assembled in the HTCC/Hep films were dissolved and detached from the film upon its exposure to an excess of charged HTCC molecules.
was less intensive (Figure S3B). However, the subsequent Hep layer was deposited even after the SPR signal dropped below the level of the previous HTCC layer (Figure 2A,B), indicating that a sufficient amount of HTCC required to establish electrostatic interactions with Hep was still present on top of the LbL film. Diffusion and redissolution processes that occur during assembly of the HTCC/Hep/CXCL12 or HTCC/Hep-mix-CXCL12 films are likely to lead to rearrangement of the layers across the film, as was shown for chitosan/HA and PLL/HA LbL assemblies.\(^{42,43}\) Although this phenomenon did not impair the assembly process in general, it represents a risk for the uncontrolled desorption of protein-containing layers from the film during multiple depositions of the HTCC/Hep/CXCL12 or HTCC/Hep-mix-CXCL12 blocks.

To restrain desorption processes during the film buildup, we introduced an HTCC/TA anchoring bilayer (TA-) to stabilize the growing HTCC/Hep assembly. TA is a polyphenol known as a physical crosslinking agent. Due to multiple hydroxyl and ester moieties present in the structure (Figure 1), TA is a weak acid and forms polyelectrolyte complexes with various polycations, including HTCC,\(^{46}\) based on hydrogen or electrostatic bonding, potentially boosting the stability of assembled LbL films.\(^{46}\) Relatively small polyphenol molecules such as TA can diffuse through hydrated LbL films and physically crosslink subsequently deposited outer layers.\(^{47}\) The black curves in Figure 2A,B show SPR sensograms for the TA-(HTCC/Hep/CXCL12)\(_n\) and TA-(HTCC/Hep-mix-CXCL12)\(_n\) assemblies with the anchoring HTCC/TA bilayer. In both assemblies, the formation of the first block was comparable to the films without the anchoring HTCC/TA bilayer. However, after the injection of the HTCC solution for the second block, the signal dropped significantly less in comparison to the signal drop observed for the corresponding films lacking the TA layer, and the stabilizing effect was maintained for the assembly of subsequent layers. Both LbL assemblies grew in an exponential-like manner (Figure 2C), which indicates diffusion of the polyelectrolyte components (here, expected HTCC) throughout the entire LbL film during the buildup process;\(^{42,48}\) this phenomenon was previously described in detail for chitosan/HA\(^{42,43}\) and PLL/HA\(^{45,48}\) LbL assemblies.

Similar to the chemokine CXCL12, the growth factors VEGF and FGF-2 belong to the family of heparin-binding proteins.\(^{17,49}\) The successful incorporation of VEGF and FGF-2 into TA-(HTCC/Hep/protein) LbL films via deposition either using a single-protein solution or into the TA-(HTCC/Hep-mix-protein) using a Hep–protein cosolution is presented in Figure 2D,E. The course of SPR patterns during the deposition of the first block of the TA-(HTCC/Hep/protein) films (Figure 2D) suggests that the character of deposition and the amount of deposited protein may depend on the protein type. No significant differences were observed in the TA-(HTCC/Hep-mix-protein) films.

One can assume a possible contribution of the proteins assembled in LbL films to the film buildup. The complexation of Hep with HTCC in PBS studied by isothermal titration calorimetry revealed a strong exothermic interaction with \(K_a = 2.4 \times 10^{9}\) M\(^{-1}\) (Table S2). However, the \(K_a\)’s for the complexation of CXCL12, FGF-2, VEGF, and TGF-\(\beta\) with Hep are one order of magnitude higher (1.3–3.2 \(\times 10^{10}\) M\(^{-1}\); Table S3); therefore, the proteins will not be released from Hep–protein complexes during self-assembly with the HTCC layers. To evaluate protein binding to HTCC, we used Lz as a model protein (because its molecular weight and isoelectric point (pI) are close to those of CXCL12, FGF-2, TGF-\(\beta\)1, and VEGF; Table S3) to follow the interactions between the pure protein or Hep–protein complex and HTCC. We recently determined \(K_a = 2.3 \times 10^{6}\) M\(^{-1}\) for the binding of Hep to Lz,\(^{36}\) which is similar to the binding of Hep to HTCC. The small quantities of Lz in the Hep–Lz complex resulted in minor changes in the titration isotherms (Figure S4), and the thermodynamic parameters for binding of Hep–Lz to HTCC are almost identical to those for pure Hep. In addition, Lz weakly binds to HTCC, with \(K_a \sim 10^3\) M\(^{-1}\) (Table S2). This suggests that the proteins under study will bind preferentially to Hep but not HTCC, and therefore, their potential to interact with HTCC in the LbL film is minimal. Furthermore, the SPR sensograms of protein-loaded films self-assembled on the anchoring layer (i.e., TA-(HTCC/Hep-mix-protein)) and without the anchoring layer (i.e., (HTCC/Hep-mix-protein)) showed that the signal decrease (indicative of the mass removal from the SPR chip surface) observed after the third and subsequent injections of the HTCC solution was markedly less intense in the case of all TA-HTCC/Hep-mix-protein films and was similar to that observed with the reference TA-HTCC/Hep films (Figure S5). Additionally, the sensograms of all HTCC/Hep-mix-protein films without the anchoring layer were highly comparable with the sensogram of the HTCC/Hep control (Figure S6). All of the above-mentioned observations allow us to assume that CXCL12, VEGF, FGF-2, and TGF-\(\beta\)1 assembled in the LbL films should not contribute to the suppression of desorption processes and thus to increased stability of the LbL films during film buildup.

### 3.3. SE Analysis of the Self-Assembly of HTCC and Hep

The assembly process in terms of the development of film thickness with an increasing number of deposited blocks was monitored via SE measurements. This study was performed on CXCL12-containing LbL films (Figure 3A). The TA-(HTCC/Hep/CXCL12) films built via the deposition of CXCL12 from a protein solution alone were significantly thicker than the TA-(HTCC/Hep-mix-CXCL12) films constructed via CXCL12 deposition from the Hep–CXCL12 cosolution. The SE thicknesses of the seven-block TA-(HTCC/Hep/CXCL12)\(_7\) and TA-(HTCC/Hep-mix-CXCL12)\(_7\) films were 69 and 35 nm, respectively. The individual protein layers did not significantly contribute to the increase in the overall thickness of the TA-(HTCC/Hep/CXCL12) films (data not shown), likely due to the very low protein concentration of 1 \(\mu\)g/mL used for the deposition and to the supposed protein diffusion through the LbL film, as was observed for the model proteins lysozyme or albumin in PLL/HA LbL films.\(^{50,51}\) Therefore, the difference in the SE film thickness might be attributed to the 40-fold higher concentration of the Hep solution used for the TA-(HTCC/Hep/CXCL12) assemblies (1 mg/mL) than the concentration of the Hep–protein cosolution used for the TA-(HTCC/Hep-mix-CXCL12) assemblies (25 \(\mu\)g/mL).

The dependence of film thickness on the number of deposited blocks is typical of exponentially growing films (Figure 3A), which correlates with the obtained SPR data (Figure 2C). The initial slow increase followed by steep exponential growth is similar to the buildup profile of chitosan/HA and chitosan/Hep LbL assemblies.\(^{34,43}\) The
The proposed phenomenon related to such assembly dynamics is the rearrangement of macromolecules during alternate adsorption of polyelectrolyte layers, caused by the mobility of HTCC molecules diffusing through the LbL assemblies.

As discussed below, data in the literature suggest that preparation conditions significantly affect the thickness of LbL films. Nonloaded chitosan/Hep assemblies that were prepared by other groups via a continuous immersion/rinsing step procedure were noticeably thinner than the HTCC/Hep LbL films prepared in our study. The dry thickness of 10-bilayer (chitosan/Hep)$_{10}$ assemblies has repeatedly been reported to be approximately 7–10 nm. Considering that the SE thickness of the individual chitosan and Hep layers is approximately 2 nm, such a low film thickness for 10-bilayer films suggests partial removal of the mass from the films during the assembly process, as we also observed in the SPR study (Figure 2A). The increased thicknesses of the TA-(HTCC/Hep/CXCL12) and TA-(HTCC/Hep-mix-CXCL12) films may be attributed to the stabilizing effect of the anchoring HTCC/TA bilayer during the process of film buildup and the strong availability of HTCC for complexation due to the positive charge independent of pH. The use of a constant pH for the assembly process can be a decisive factor. LbL films based on chitosan can be prepared from a chitosan solution of pH 4–5, at which chitosan with a pK$_a$ of approximately 6.3 is sufficiently protonated to be water-soluble, and from a polyanion solution of pH 7.4 (with the pK$_a$ of the polyanion typically below pH 4, depending on the polyanion type), which is a pH suitable for protein incorporation. However, fluctuations in pH during film preparation may result in a decreased stability of films composed of weak polyelectrolytes, such as chitosan, due to shifts in the overall net charge of the polyelectrolytes forming the LbL films. Indeed, (Hep/chitosan)$_8$ or (Hep/chondroitin sulfate)$_8$ LbL films prepared from polyelectrolyte solutions of pH 4 had a thickness of approximately 27 nm. Furthermore, the technical parameters of the coating, such as the rinsing protocol or the drying step, can also significantly influence the film thickness. For example, Juang et al. reported that (chitosan/Hep)$_5$ films prepared at pH 5 had an SE thickness of 80 nm. In this case, the rinsing step after each deposition step was performed in a very small volume of 1 mL for a short time of 60 s (relative to the procedures described in other studies). The short rinsing step can result in less effective removal of loosely attached molecules of the deposited polyelectrolyte and thus in more extensive formation of polyelectrolyte complexes on the film surface, leading to increased film thickness. Exponentially growing (chitosan/Hep)$_n$ films with thicknesses of 34 and 98 nm were also obtained when the surfaces were dried under a stream of N$_2$ after each rinsing step. As has been reported for several types of LbL films, the drying process leads to the

![Figure 3](image-url)

**Figure 3.** Spectroscopic ellipsometry evaluation of LbL films: (A) dependence of the ellipsometric thickness of the TA-(HTCC/Hep/CXCL12) (square) and TA-(HTCC/Hep-mix-CXCL12) (triangle) LbL films on the number of deposited blocks. (B) Disintegration of the TA-(HTCC/Hep/CXCL12)$_{10}$ (square) and TA-(HTCC/Hep-mix-CXCL12)$_{10}$ (triangle) LbL films in PBS at 37 °C. The data are expressed as mean ± SD (n = 3).

![Figure 4](image-url)

**Figure 4.** XRR analysis: (A) XRR profiles of the TA-HTCC/Hep/CXCL12-HTCC/HEP) film at various deposition steps. The building of the LbL film started with the anchoring HTCC/TA layer (denoted as TA-, black pattern), and the subsequent layers were deposited. The curves are shifted vertically for clarity. (B) Scattering length density profile for the TA-HTCC/Hep (red pattern) and TA-HTCC/Hep/CXCL12 (a blue pattern) LbL films. LbL films were deposited on silica substrates.
reorientation of the polyelectrolyte molecules, including reorientation of unneutralized sites with unsaturated charges in the upper surface layer in the air. Such reorganization of macromolecules facilitates film growth, leading to thicker films. \(^{63-65}\) Nevertheless, automatically controlled LB coaters used to modify a large number of substrates do not typically allow a drying process to be applied.

The disintegration profiles of the TA-(HTCC/Hep/CXCL12\(_{10}\)) and TA-(HTCC/Hep-mix-CXCL12\(_{10}\)) LB films incubated in PBS for 35 days are presented in Figure 3B. The films exposed to physiological pH, ionic strength, and a temperature of 37 °C exhibited an initial abrupt decrease in thickness during the 1st week of incubation. Further exposure resulted in a moderate, nearly linear thickness decrease of up to 52 and 41% of the initial value for TA-(HTCC/Hep-mix-CXCL12\(_{10}\)) and TA-(HTCC/Hep/CXCL12\(_{10}\)) films, respectively, after 5 weeks of exposure. A comparable 35% loss in thickness was reported for (Hep/chitosan)\(_6\) assemblies after a two-week incubation. \(^{61}\)

### 3.4. X-ray Reflectivity Evaluation of the Thickness of HTCC/Hep LbL Films

While both the SPR and SE analyses convincingly point to the successful formation of LB structures, they are incapable of probing the possible intermixing and creation of interpenetrating networks between the individual components of the LB assembly. To investigate the possible intermixing of the deposited layers during film assembly, we utilized X-ray reflectivity (XRR) measurements. The obtained XRR curves are presented in Figure 4. To more deeply understand the profiles, the reflectivity curves were refined using a GenX software. \(^{66}\) Due to the many fitting parameters, we postulated that the bottom layers were not affected by the deposition of the upper layers, and due to the deviation in the deposition process, the parameters were fitted within very narrow constraints.

The oscillation minima observed in the XRR profiles shift to lower angles (2\(\theta\) values) with an increasing number of deposited layers, indicating an increase in film thickness. The fitting results showed that the first HTCC layer, a component of the anchoring HTCC/TB bilayer, had the largest thickness of 25.12 Å and a roughness of only 3.77 Å, and its bulk density was maintained (Figure 4A, the TA sample, a black pattern). The subsequent deposition of the TA layer was not as effective, resulting in only a 5.00 Å thick layer, with a similar roughness value. The density of the TA layer approached the value usually observed in bulk. After deposition of the first HTCC/ Hep bilayer (Figure 4A, the sample TA-HTCC/Hep, a red pattern), the deposited HTCC layer already exhibited different parameters; the determined thickness of 8.00 Å was much lower than that of the first HTCC layer and showed a very high roughness that was comparable to the layer thickness. Moreover, the density decreased to a value of 1.2 g/cm\(^3\). Furthermore, we can expect that the properties of the Hep layer are the key to effective deposition of the chemokine CXCL12. Immersion of the TA-HTCC film into the Hep solution and subsequent Hep deposition appeared to reduce the roughness of the upper HTCC layer from 10.00 to 6.80 Å, and the density increased nearly to the value of the bulk HTCC while all other parameters were maintained. The thickness of the deposited Hep layer was 10.02 Å, again with a comparable roughness value and with a density twice as low as that in the bulk. The corresponding scattering length density (SLD) profile of the TA-HTCC/Hep film is shown in Figure 4B (a red pattern).

Overall, the obtained data indicate that the interface between the deposition of subsequent layers is rather rough; however, the surface becomes smoother upon subsequent immersion. The SLD profile for the TA-HTCC/Hep/CXCL12 sample (Figure 4B, a blue pattern) supports this assumption. The thickness of the deposited CXCL12 layer was rather low, specifically, 5.04 Å, likely due to the low concentration of the CXCL12 solution (1 \(\mu\)g/mL) used for deposition (Figure 4A, a blue pattern). The mean roughness value of 11.94 Å was larger than the layer thickness, with a high error in roughness estimation, which might be the result of the interpenetrating process. In addition, the interface of the second HTCC layer became smoother with a roughness of 3.75 Å. This suggests that penetration of the solvent through the film occurs, causing additional shrinkage of the second HTCC layer. Indeed, the density of the HTCC and Hep layers slightly increased, which appears as an increase in the SLD profile (Figure 4B, red pattern).

Deposition of the subsequent HTCC/Hep bilayers increased the film thickness. The thickness of the second Hep layer was 4.50 Å and was comparable with that of the previous Hep layer. The deposition of HTCC reduced the roughness of the previous Hep layer and maintained its thickness, indicating that the formed layer stuck well. Furthermore, the high values of the calculated roughness for the subsequent Hep and HTCC layers (12–14 Å) again indicate the penetration of Hep or HTCC into the CXCL12 layer. We obtained an overall film thickness of approximately 70 Å for the TA-HTCC/Hep/CXCL12-(Hep-HTCC)\(_3\) sample and 80 Å for the TA-HTCC/Hep/CXCL12-(Hep-HTCC)\(_5\) sample. The lower thickness values than the values obtained by SE can be attributed to the differences in the methods and assumptions used for fitting the data. However, the observed trends within the XRR analysis results are similar to those obtained from the SPR and SE analyses. The excess of fitting parameters and material absorption makes it impossible to obtain a reasonable fit parameter for the TA-HTCC/Hep/CXCL12-(Hep-HTCC)\(_3\) and TA-HTCC/Hep/CXCL12-(Hep-HTCC)\(_5\) samples. Even though further deposition of the layers is beyond a reasonable evaluation, the XRR data clearly point to the formation of interpenetration networks of the Hep or HTCC and CXCL12 layers.

In addition to the XRR method, diffusion processes within LbL films during the self-assembly process, which lead to more complex film architectures than a distinct layered structure, have been investigated using several techniques. Inward and outward diffusion of polyion components through PLL/HA and chitosan/HA LbL films during buildup was previously demonstrated via confocal laser scanning microscopy using fluorescence-labeled PLL or chitosan. \(^{42,45}\) PLL/HA films with a thickness of a few micrometers were used for these studies. Dynamic processes within LbL films have also been analyzed via optical waveguide light mode spectroscopy. Variations in the refractive index of the film in the vicinity of the film/waveguide interface during the buildup of PLL/HA and PLL/(L-glutamic acid) LbL films were attributed to out-of-plane diffusion of polyelectrolytes through the film/solution interface, propagating throughout the entire film. \(^{46}\) Finally, fluorescence resonance energy transfer and total internal reflection fluorescence techniques have demonstrated that the polymers deposited within chitosan/Hep LbL films freely interdiffuse in
the transverse direction, destroying any discrete layer-by-layer organization within the film.

3.5. Topography of HTCC/Hep LbL Films. The changes in surface topography during film buildup were evaluated using AFM. Although dry-state AFM analysis does not fully reflect the native state of surfaces due to dehydration of the swollen LbL film, this approach allows us to standardize sample preparation and avoid the changes in surface topography due to the presumed disintegration of LbL films kept swollen (Figure 3B) prior to and during AFM analysis. Figure 5 shows the scans of TA-(HTCC/Hep/CXCL12)$_n$ and TA-(HTCC/Hep-mix-CXCL12)$_n$ films consisting of one, three, five, and seven blocks in the dry state. The surfaces of both types of LbL films with one deposited block were covered by evenly

Figure 5. AFM images of TA-(HTCC/Hep/CXCL12)$_n$ and TA-(HTCC/Hep-mix-CXCL12)$_n$ films consisting of one, three, five, and seven blocks. The film topography was measured in air. The $R_{\text{rms}}$ value measured for a particular surface is displayed in the upper-right corner of the image. Image size: $2.5 \mu m \times 2.5 \mu m$; the $z$-scale is given to the right side of the scans.

Figure 6. In vitro release of proteins from 15-bilayer HTCC/Hep LbL films: (A) cumulative release of CXCL12 from films with the HTCC/TA and PEI anchoring bilayer; CXCL12 was loaded as individual layers (i.e., TA-(HTCC/Hep/CXCL12)$_{15}$ and PEI-(Hep/CXCL12/HTCC)$_{15}$) or as layers mixed with Hep (i.e., TA-(HTCC/Hep-mix-CXCL12)$_{15}$ and PEI-(Hep-mix-CXCL12/HTCC)$_{15}$). (B) Cumulative release of TGF-$\beta$, CXCL12, FGF-2, and VEGF from TA-(HTCC/Hep-mix-CXCL12)$_{15}$ films. (C) Actual release of TGF-$\beta$, CXCL12, FGF-2, and VEGF proteins from TA-(HTCC/Hep-mix-protein)$_{15}$ films. The data are expressed as mean ± SD (5 samples).
distributed, small, round-shaped objects that represent surface domains covered with the first HTCC/Hep coacervates. It has also been proposed that such “spheres” observed at the beginning of the chitosan/HA assembly likely represent single-coiled chitosan molecules. In the case of the TA-(HTCC/ Hep/CXCL12) \(_2\) films, the spheres increased in size, and the surfaces were almost fully covered by separated islet-like objects that were already partially interconnected, whereas the TA-(HTCC/Hep-mix-CXCL12) films exhibited granular structures at the later phase of film buildup. The observed islet structures were attributed to the formation of coacervates of oppositely charged polyelectrolytes on the film surface. With an increasing number of deposited blocks, the separated islets merged into longer shapeless structures, leading to a vermiculate morphology. This transition appears to be reflected in the increase in surface roughness, \(R_{\text{rms}}\). A similar evolution in surface topography from circular structures to larger vermiculate structures was observed for chitosan/HA assemblies. AFM scans showed a slower evolution in the surface topography of the TA-(HTCC/Hep-mix-CXCL12) assembly. The transition from isolated islands to vermiculate morphology occurred from the deposition of the 5th block for TA-(HTCC/Hep/CXCL12) films and the 7th block for TA-(HTCC/Hep-mix-CXCL12) films, and it seemed to correlate with the onset of the exponential growth in film thickness (Figure 3A). The observed changes in topography are characteristic of various polyelectrolyte LbL films, typically exponentially growing films, and were also reported for LbL assemblies composed of chitosan or modified chitosan and HA, poly(acrylic acid), or poly(γ-glutamic acid).  

### 3.6. In Vitro Protein Release

The in vitro release of CXCL12, FGF-2, VEGF, and TGF-β1 from the HTCC/Hep LbL films was performed in PBS with 0.1% BSA and 0.02% NaCl. The main study was carried out on CXCL12-containing LbL films. Figure 6A presents the one-week cumulative CXCL12 release from TA-(HTCC/Hep/CXCL12) \(_2\) and TA-(HTCC/Hep-mix-CXCL12) \(_2\) LbL films. Overall, both assemblies exhibited a rapid initial release for the first four hours, then the release slowed, and after 1 week, a small but gradual release of approximately 30 pg mL\(^{-1}\)cm\(^{-2}\) occurred until the end of the study (Figure S7A). A comparable CXCL12 release profile was reported for poly(γ-glutamic acid)/chitosan LbL films.  

Protein deposition using the CXCL12 solution alone was less beneficial than deposition using the Hep-CXCL12 coacervation. After 1 week of incubation, the TA-(HTCC/Hep/CXCL12) \(_2\) films released 32% less CXCL12 than the TA-(HTCC/Hep-mix-CXCL12) \(_2\) films. We can speculate that when Hep molecules are complexed with the upper HTCC layer of the growing films, rearrangements in the film structure occur, and thus Hep molecules may be less available for subsequent binding with free CXCL12 molecules than the binding of Hep to CXCL12 directly in their coacervation. The molar Hep/CXCL12 ratio in the coacervation was approximately 12:1, indicating a high probability that most of the Hep chains bind the CXCL12 molecule, but a sufficient number of negatively charged groups is still available to complex with the upper HTCC layer of the growing TA-(HTCC/Hep-mix-CXCL12) films.  

Polyethyleneimine (PEI) is a weak polycation with hydrophilic-binding sites and hydrophobic chains and efficient adsorption to various surfaces. PEI is frequently used as the first layer to anchor LbL films on surfaces of different origins. Therefore, we compared the release of CXCL12 from the HTCC/Hep LbL films prepared with the HTCC/TA and PEI anchoring layers (Figure 6A). The films formed on the HTCC/TA anchoring bilayer released 3 and 4 times more CXCL12 from the TA-(HTCC/Hep/CXCL12) \(_5\) and TA-(HTCC/Hep-mix-CXCL12) \(_5\) films, respectively, than that released from the corresponding LbL films assembled on the PEI anchoring layer. Furthermore, the increase in thickness of the PEI-(Hept/HTCC) films with an increasing number of deposited bilayers was lower than the increase observed for the TA-(HTCC/Hep) films (Figure S8). We can therefore speculate that more pronounced desorption processes occurred during the assembly of PEI-(Hept/HTCC) films, which resulted in a lower number of protein-containing layers and hence less protein release. Thus, the SE and release experiment results indirectly suggest a stabilizing effect of the HTCC/TA bilayer on the film structure during film buildup, which is consistent with the outcomes of the SPR experiments (Figure 2A,B).  

TGF-β1, VEGF, and FGF-2 proteins were incorporated into the LbL assemblies in the form of mixed Hep–protein layers. The four-week cumulative and actual release from the TA-(HTCC/Hep-mix-protein) \(_3\) LbL films are presented in Figure 6B,C. The total protein amount decreased in the order TGF-β1 > FGF-2 > CXCL12 > VEGF. The release profiles of CXCL12, TGF-β1, and VEGF exhibited a typical pattern reported for the release kinetics of proteins from LbL films; in particular, an initial fast release occurred in the first eight hours, followed by a slower release for as many as 2 days. The films released 95% of the total released TGF-β1 and CXCL12 amount and 86% of the total released VEGF amount within 48 h. However, in comparison with the literature, a gradual, albeit low, delivery of approximately 10, 50, and 60 pg mL\(^{-1}\)cm\(^{-2}\) of TGF-β1, CXCL12, and VEGF, respectively, continued for the following 3 weeks (Figure S7B). The FGF-2 release profiles differed from the CXCL12, TGF-β1, and VEGF profiles. No considerable initial release was observed, with only 30% of the total FGF-2 amount released during the first 48 h. Importantly, the release then remained almost linear, with approximately 1000 pg mL\(^{-1}\)cm\(^{-2}\) released during the 2nd week and 1500 pg mL\(^{-1}\)cm\(^{-2}\) (i.e., 9.2 pg mL\(^{-1}\)h\(^{-1}\)cm\(^{-2}\)) released during the next 2 weeks, reaching a total release amount of approximately 7 ng mL\(^{-1}\)cm\(^{-2}\).  

It can be assumed that the observed differences in release profiles may be related to different properties of particular proteins. Selected characteristics of the proteins under study available in the literature, i.e., molecular weight, size, isoelectric point, affinity constant \(K_a\) for Hep (reflecting the strength of the interactions), and association (\(k_a\)) and dissociation (\(k_d\)) rate constants (reflecting the kinetics of protein–Hep complex formation), are listed in Table S3. No clear relationship between the protein characteristics and the observed release profiles was identified. All proteins under study are positively charged under physiological conditions. The \(pH\) decreases in the order CXCL12 > FGF-2 > TGF-β1 > VEGF, and the molecular weight decreases in the order VEGF > TGF-β1 > FGF-2 > CXCL12, but these trends do not follow the trend in total protein release, i.e., TGF-β1 > FGF-2 > CXCL12 > VEGF. Concerning the molecular weight and size of protein molecules, the literature data only show that most of the evaluated proteins bind to heparin with a higher affinity than their fragments, and thus, no conclusion to our experiments can be applied. Furthermore, the protein release from HTCC/
Hep-mix-protein LbL films may also be affected by the character of protein—Hep interactions. The \( K_s \) values of \( 10^7 \) M\(^{-1} \) are very similar for all four proteins and indicate very strong binding of proteins to Hep. However, these interactions are not purely electrostatic, hydrogen bonding, van der Waals forces, and hydrophobic interactions also contribute, the extent of which depends on the specific protein.\(^{18} \) In addition, heparin-binding proteins usually have two Hep-binding sites; however, the minimum number (ranging from tetra- to tetradecasaccharide residues) and type of the sulfation pattern of saccharide residues in the Hep chain, which provide strong binding to individual proteins, differ.\(^{19} \) Numerous approaches have been used to study glycosaminoglycan-protein interactions (e.g., solid-phase and filter-binding assays, affinity chromatography, electrophoretic techniques, SPR, X-ray crystallography, NMR spectroscopy, and molecular modeling) as reviewed by Peysselon et al.\(^{69} \) Nevertheless, these approaches are generally used to analyze binary systems, typically in equilibrium, and require milligram quantities of interacting species for analysis, which is not the case for multicomponent LbL films. Therefore, in the absence of methods to analyze interactions between Hep, HTCC, and protein directly self-assembled in LbL films, we can only assume the cumulative effect of various parameters on protein release from TA-HTCC/Hep-mix-protein LbL films.

Direct comparison of LbL films reported in the literature in terms of the released protein amount is a challenging task due to differences in experimental designs and the lack of data on the released amount relative to the sample area. However, basic characteristics, such as the release profile and duration of release from LbL films containing chitosan or Hep, can be partially discussed. Naves et al. reported on PEI-(chitosan/ Hep), reservoirs when FGF-2 was deposited from a cosolution with chitosan.\(^{11} \) The films were composed of three and six bilayers and released FGF-2 in very low doses for 7 days, and then, the release moderately increased during the next week. The released amount was one order of magnitude lower than that observed in our study, likely due to the small number of bilayers. LbL films of chitosan and poly(β-amino esters) (PMLA), i.e., (chitosan/PMLA/FGF-2/PMLA), consisting of 20 and 60 tetralayers assembled at pH 5, sustainably released FGF-2 for 2 days, and then the release markedly slowed until 10 days.\(^{10} \) The cumulative release from 20-tetralayer assemblies was approximately 7 ng/cm\(^2\), comparable with the FGF-2 release in our study. CXCL12 and interferon-γ were deposited from a cosolution with chitosan at pH 5 during the preparation of poly(γ-glutamic acid)/chitosan LbL films and were almost completely released within 24 h. The release below 2 ng mL\(^{-1}\)/cm\(^2\) can again be attributed to the small number of deposited bilayers.\(^{58,71} \) LbL films of chitosan and epidermal growth factor, i.e., (chitosan/EGF), released the protein for four days with a typical high initial release of 76% within 24 h and a total release amount of 5.5 ng/cm\(^2\).\(^{72} \) Tetralayer films of poly(β-amino esters) (PBAE) and Hep, i.e., (PBAE/Hep/FGF-2/Hep), released approximately 5 ng/cm\(^2\) of FGF-2 after 12 h, and then the release stopped, whereas the thicker (PBAE/Hep/FGF-2/Hep) \(_{10}\) assembly continuously released up to 40 ng/cm\(^2\) protein during a 3-day incubation.\(^{73} \) LbL films composed of Hep and a blend of collagen (Col) and PBAE as a polycation layer, i.e., (PBAE + Col/Hep/FGF-2), exhibited a high initial release of FGF-2 during the first hours.\(^{75} \) The high film thickness of several hundred nanometers likely resulted in a relatively high amount of FGF-2 released, i.e., 10.56 ng/cm\(^2\). (Col/Hep) \(_{3}\) LbL films assembled at pH 4 on polylactide nanofibers released nerve growth factor for approximately 48 h. The release was prolonged to 2 weeks with increasing film thickness, likely due to the high surface area and complex three-dimensional morphology of the nanofiber scaffold.\(^{29} \)

### 3.7. Bioactivity of the Released CXCL12

The TA-(HTCC/Hep) \(_{15}\) LbL films not loaded with protein did not exhibit cytotoxic properties; there was no significant difference in the viability of MG-63 cells (selected as model adherent cells) cultivated with the TA-(HTCC/Hep) \(_{15}\) LbL films and the control (Figure 7A). The observed biocompatibility corresponds to the good biocompatibility of LbL films assembled at pH 4 on polylactide nanofibers released nerve growth factor for approximately 48 h. The release was prolonged to 2 weeks with increasing film thickness, likely due to the high surface area and complex three-dimensional morphology of the nanofiber scaffold.\(^{29} \)

![Figure 7](https://example.com/fig7.png)

**Figure 7.** In vitro evaluation of TA-(HTCC/Hep) films. (A) Viability of MG-63 cells cultivated with TA-(HTCC/Hep) \(_{15}\) LbL films for 72 h. Three independent experiments were performed in triplicate; one-way ANOVA using Dunnett’s multiple comparison post-tests, **** refers to \( p < 0.0001 \). (B) Migration of Jurkat cells stimulated by CXCL12 released from TA-(HTCC/Hep/CXCL12) \(_{15}\) and TA-(HTCC/Hep-mix-CXCL12) \(_{15}\) LbL films and by soluble CXCL12 as a positive control. Values represent the number of migrated cells expressed as a % of the negative control (without CXCL12 added), which was set as 100%. The TA-(HTCC/Hep/CXCL12) \(_{15}\) and TA-(HTCC/Hep-mix-CXCL12) \(_{15}\) samples contained 2.5 and 3.7 ng of released CXCL12 (ELISA evaluation). The data are expressed as mean ± SD (\( n = 5 \)). One-way ANOVA using Tukey’s multiple comparisons post-test method was used for statistical analysis. Indicators above the columns show significant differences compared to the samples identified with the number indicated; * refers to \( p < 0.05 \) **** to \( p < 0.0001 \).
composed of HTCC and tannic acid\textsuperscript{32} and to the negligible cytotoxicity of several HTCC-based systems proposed for gene and vaccine delivery, heparin removal from the blood or wound dressing applications, as reviewed by Freitas et al.\textsuperscript{26}

According to the literature, growth factors released from LbL films generally effectively influence cellular responses,\textsuperscript{9,32,67,11} indicating that their function is preserved during film fabrication. In our work, we demonstrated the bioactivity of the proteins released from LbL films on CXCL12. The homeostatic chemokine CXCL12 is known as an efficient chemotactant for various cell types, including inflammatory cells, hematopoietic stem cells, and endothelial cells,\textsuperscript{74} and its role in the recipient’s immune response, angiogenesis, and wound healing processes has recently become of great interest in TE applications. In our in vitro study, we evaluated the migration of a human T lymphocyte cell line (Jurkat cells) upon stimulation with CXCL12 using a cell chemotactic migration assay. Preliminary experiments showed that the experimental history of the cultivation medium significantly affected cell migration (Figure S2B). The migration of Jurkat cells decreased by approximately 30% when the cultivation medium was incubated in 5% CO\textsubscript{2} at 37 °C for 48 h, which were the conditions used to prepare the samples for migration experiments. This could result from a partial deactivation of proteins, such as growth factors present in the serum added to the culture medium, upon incubation. Therefore, to avoid inaccurate results, the cultivation medium exposed to 5% CO\textsubscript{2} for 48 h was also used for the control samples. Unexpectedly, Jurkat cells migrated slightly more in the medium incubated in the presence of TA-(HTCC/Hep)\textsubscript{15} films than in the medium incubated alone and comparably with the cells that migrated in the fresh medium. Figure 7B presents the migration of Jurkat cells upon stimulation with CXCL12 released from the TA-(HTCC/Hep/CXCL12)\textsubscript{15} and TA-(HTCC/Hep-mix-CXCL12)\textsubscript{15} films. Jurkat cells migrated significantly more than in the control and reached almost 600% of the control in the TA-(HTCC/Hep-mix-CXCL12)\textsubscript{15} samples. Cell migration also increased with increasing CXCL12 levels (Figure 6A), which was associated with the ability of Jurkat cells to respond to CXCL12 in a dose-dependent manner.\textsuperscript{74} In addition, it was previously reported that approximately 4 times less CXCL12 than the amount released from the LbL films in our study successfully stimulated the migration of mesenchymal stem cells and mouse myoblast cells,\textsuperscript{13,68} suggesting that both TA-(HTCC/Hep/CXCL12)\textsubscript{15} and TA-(HTCC/Hep-mix-CXCL12)\textsubscript{15} films release CXCL12 in sufficient quantities to influence the response of different cell types.

The proposed TA-(HTCC/Hep) LbL films that release heparin-binding proteins meet important requirements for the delivery of bioactive proteins, such as a lack of cytotoxicity and effective protein delivery, also resulting from film fabrication under non-denaturing conditions. The SPR analysis showed that careful in situ monitoring of the self-assembly can provide useful information about physical phenomena occurring during the buildup process, which offers the possibility of optimizing conditions for the modification of real substrates to obtain a functional coating. Additionally, differences in release profiles determined in our study point to the need to carefully evaluate specific proteins in any proposed delivery system, suggesting that the use of model proteins, such as albumin or lysozyme,\textsuperscript{24,76} should be limited to preliminary studies only. We envisage that different release profiles could pave the way toward LbL coatings with the capacity for combined and sequential release of proteins, thus concomitantly targeting a possible timeline to induce specific cellular responses.

4. CONCLUSIONS

In the present study, we report on a process for assembling polyelectrolyte LbL films composed of quaternized chitosan (HTCC) and heparin (Hep) and the ability of these films to load and deliver heparin-binding proteins, i.e., CXCL12, FGF-2, VEGF, and TGF-β1.

Water-soluble HTCC and Hep successfully formed self-assembled multilayer films loaded with proteins under physiological, non-denaturing for proteins conditions, as shown by SPR, SE, and XRR evaluations. The use of an anchoring bilayer consisting of HTCC and tannic acid led to a reduction in desorption during film assembly and, consequently, to increased film thickness and thus higher protein loading. Protein release depended on the manner in which the bioactive proteins were incorporated into the LbL films, that is, either as individual layers or as an integral part of the Hep layers. The TA-(HTCC/Hep-mix-CXCL12)\textsubscript{15} films loaded with the protein via deposition of a Hep–protein cosolution released 32% more CXCL12 than the TA-(HTCC/Hep/CXCL12)\textsubscript{15} films loaded with CXCL12 using a protein solution alone. In addition, both LbL films released 3 and 4 times more CXCL12 than the corresponding films assembled on a PEI anchor layer, which is the anchoring layer typically used to fabricate LbL coatings on different surfaces.

The release profiles from TA-(HTCC/Hep-mix-protein)\textsubscript{15} films depended on the protein loaded. The total released protein amount decreased in the order TGF-β > FGF-2 > CXCL12 > VEGF. Furthermore, VEGF, CXCL12, and TGF-β exhibited a typical high initial release phase, followed by a low gradual long-term release, whereas FGF-2 was sustainably released in an almost linear manner for 4 weeks. The LbL assemblies were not toxic to model MG-63 cells and Jurkat cells, and the proteins retained their bioactivity, as demonstrated by the effective induction of in vitro chemotaxis of T-lymphocytes by CXCL12 released from the films. The ability to deliver heparin-binding growth factors and chemokines, a good biocompatibility, and possibility of deposition on various biomaterial surfaces make HTCC/Hep LbL films a promising tool for the bioactive surface modification of various medical devices.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.2c00926.

Experimental part regarding the synthesis of N-[2-(2-hydroxy-3-trimethylammonium)propyl]chitosan; modification of SPR chips with MESNA; modification of silica wafers and glass substrates; film characterization; ITC evaluation of interactions between lysozyme, Hep or Hep–lysozyme, and HTCC in PBS solution; protein release; biocompatibility study and cultivation of Jurkat cells; results regarding the conditions of HTCC synthesis and degree of substitutions; \textsuperscript{1}H NMR spectra of HTCC; optimization of conditions for the cell migration assay; SPR sensograms of reference HTCC/Hep and HTCC/Hep/CXCL12 LbL films; ITC isotherms and thermodynamic parameters of Hep and
Hep–lysozyme interactions with HTCC; characteristics of proteins used for study; SPR sensograms of individual TA-HTCC/Hep–protein; HTCC/Hep–protein (without the anchoring HTCC/TA layer) and reference TA-HTCC/Hep LbL films; summary of SPR sensograms of HTCC/Hep–protein and reference HTCC/Hep LbL films; actual protein release data; and ellipsometric thickness of LbL films assembled on the PEI anchoring layer (PDF)

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T.U. and D.K. contributed equally to this work. The manuscript was written by T.U., D.K., and A.Z. Main revisions were made by D.K. and O.P.G., with contributions from the rest of the authors. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

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