Nanostructured highly-swollen hydrogels: Complexation with amino acids through copper (II) ions

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ABSTRACT

We report on the complexation of highly swollen poly(methacrylic acid) (PMAA) nanothin hydrogel films with copper (II) ions and the stability of these (PMAA)-Cu²⁺ complexes in amino acid solutions. These networks are produced by selective cross-linking of PMAA with adipic acid dihydrazide (AAD) and ethylenediamine (EDA) in hydrogen-bonded spin-assisted layer-by-layer films. The swelling of the hydrogels **at higher pH values** is significantly suppressed after introducing copper (II) ions and can be restored by treatment with amino acid solutions including serine, threonine, arginine, histidine and glutamine. The **hydrogel** swelling recovery is controlled by Cu²⁺ removal from the hydrogel which is regulated by the strength of [(PMAA)_n-Cu²⁺] complex bonding and is dependent on the amino acid type, concentration and the hydrogel's cross-linker. The use of AAD as a cross-linker allows for selective **binding** and recognition of arginine among other amino acids. **The special affinity of the [(PMAA)₁₆-AAD-Cu²⁺] hydrogel complex to arginine is due to the formation of ternary (hydrogel-copper-arginine) complexes stabilized by electrostatic interactions between the carboxylic groups of PMAA and the guanidinium side group of arginine.** Our study provides new aspects of competing interactions between polyelectrolyte networks, metal ions, and amino-acids and opens prospects for developing novel analyte-responsive biosensors.

Keywords: pH-responsive multilayer hydrogels, copper (II) ions, amino acids

1. Introduction

Stimuli-responsive hydrogels capable of reversible volume changes triggered by guest molecules have gained considerable attention during recent decades due to their potential application in sensing [1-7]. Being modified with specific binding sites such as ions, hydrophobic groups or small organic molecules, the networks can change the volume in the presence of various analytes [8-11]. In this respect, detecting amino acids with hydrogels is of special interest since they play vital roles in biological organisms being building blocks for proteins and peptides, and as neuro-mediators [12-14]. For example, chitosan networks modified with benzoic acid have been shown to selectively increase in volume by 60% in histidine solutions [15]. However, after incorporation of 9-antrylaldehyde the chitosan hydrogels were sensitive to the presence of methyl esters of lysine, alanine and phenylalanine solutions [16].

One of the interesting features of amino acids is their metal-complexing capability, which forms chelates through the amino and/or carboxylic groups [17,18]. Poly(methyl methacrylate) (PMMA)-diethylenetriamine-dodecylamine gel was shown to provide strong affinity toward Cu^{2+} and Zn^{2+} due to the metal chelating ability of the diethylenetriamine [19]. In another example, loading PMMA gel with Cu (II) ions changed its volume expansion in the presence of glycine and histidine as compared to the unloaded hydrogel [20]. Acrylamide-tetracycline photonic crystal hydrogels modified with Cu^{2+} exhibited a red-shift of the diffraction wavelength with a magnitude dependent on the concentration of glycine [21]. The high affinity of amino acids towards Cu^{2+} has been applied for quick metal ion removal from copper (II)-based polymer complexes [22-26]. The ability of amino acids to remove copper Cu^{2+} ions from the Cu^{2+} -polymer complexes was found to be strongly dependent on the concentration and type of amino acids. [21-27].

The majority of studies on the interaction of copper (II)-based complexes with amino acids have focused mainly on polymer solutions and bulk hydrogels [16,20,22-26,28]. However, these ‘thick’ networks generally demonstrate a slow diffusion-based response (from minutes to hours) and inhomogeneous swelling due to variations in the cross-link density within the hydrogels [3,15]. The swelling rate of a gel is inversely proportional to the square of the sample size; thus, decreasing the size of polymer gels to a nanometer scale would significantly improve the overall response time and reduce it to seconds [29].

Layer-by-layer (LbL)-derived hydrogels offer nanothin networks with thickness, structure, cross-link density, and properties controlled at the nanoscale [30-33]. These multilayer hydrogels are produced by the cross-linking of multilayers either during or post-assembly and undergo reversible stimuli-triggered swelling/shrinking provided by functional groups not involved in covalent binding [32-41]. In contrast to traditional hydrogel coatings, LbL-derived networks can be deposited on substrates of various shapes and morphology [42-48]. These nanothin hydrogels are capable of fast and dramatic volume change and can host various types of molecules and ions [49-54]. Sukhishvili *et al* reported on the ability of (PMAA) networks to reversibly absorb large amount of proteins and peptides [40,55]. We have recently shown that poly(4-vinylpyridine) (PVP) nanothin hydrogels produced from PVP/poly(methacrylic acid) (PVP/PMAA) multilayers exhibit a fast (within seconds) and drastic 10-fold reversible swelling when pH is switched from neutral to acidic [49].

Despite the intriguing properties of stimuli-responsive LbL-derived hydrogels, little is known about their complexation with metal ions. Several groups investigated the interaction of Cu^{2+} with poly(carboxylic acids) films, brushes, and electrostatically-bound multilayers [56-61]. Sukhishvili *et al.* found that Cu^{2+} binding to poly(acrylic acid)/branched polyethyleneimine

(PAA/BPEI) multilayer films was due to two driving forces: ionic pairing due to carboxylic groups and chelating ability of the -NH- groups of BPEI [56]. A similar Cu^{2+} -binding mechanism was reported later by Zacharia *et al.* for the (BPEI-Cu(II)/PAA) system [58]. In that work, the metal ions in the film worked as an additional cross-linker between the amine groups of BPEI and the carboxylic groups of PAA. However, there are no studies on the interaction of copper (II) ions with nanothin LbL-based PMAA hydrogels. Moreover, the stability of hydrogel-metal ion complexes in the presence of other species, including amino acids, has not been explored. The limited knowledge is probably associated with synthetic and instrumental challenges to produce and characterize nanothin networks of controlled architecture and swelling. An understanding of that complexation and complex stability would open new avenues for developing network-based biosensors.

We have recently produced highly swollen $(\text{PMAA})_n$ hydrogels (n is a layer number), which swell to 18 times their dry thickness in $\text{pH}=7.5$, indicating 90 v/v % of water in the swollen ionic film [50]. Those films were obtained from well-stratified hydrogen-bonded PMAA/poly(N -vinylpyrrolidone) $(\text{PMAA/PVPON})_n$ multilayer precursors via a spin-assisted LBL approach [50].

In the current study we report for the first time on the formation and properties of $[(\text{PMAA})_n \text{ hydrogel-Cu}^{2+}]$ complexes with a special focus on stability of these complexes in amino acid solutions. We are motivated by the fact that recognition of amino acids is of increasing importance for fundamental research and practical applications [12-14]. We explore the ability of highly swollen $(\text{PMAA})_n$ hydrogel films to bind Cu^{2+} and the stability of the produced complexes in the presence of histidine, arginine, glutamine, serine and threonine. The role of hydrogel type and amino-acid concentration on the strength of $[(\text{PMAA})_n\text{-Cu}^{2+}]$ bonding are

elucidated. Loading of Cu^{2+} into PMAA hydrogel films is tested with UV-vis spectroscopy, EDS, *in situ* ATR-FTIR, *in situ* ellipsometry, and AFM. We demonstrate that the use of adipic acid dihydrazide (AAD) as a hydrogel cross-linker allows for selective binding and recognition of arginine among the other investigated amino acids. Arginine is crucial for cell division, functioning of the immune system, wound healing and hormone release [62,63]. Our work introduces new fundamental aspects of competing interactions between polyelectrolyte networks, metal ions, and amino-acids and opens prospects for developing novel analyte-responsive biosensors.

2. Experimental section

2.1 Materials

Poly(methacrylic acid) (PMAA, average M_w 100,000 g mol^{-1}), poly(N-vinylpyrrolidone) (PVPON, average M_w 55,000 g mol^{-1} and M_w 2,500 g mol^{-1}), Poly(allylamine hydrochloride) (PAH, average M_w 58,000 g mol^{-1}) mono- and dibasic sodium phosphate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, 99.5% titration), ethylenediamine (EDA), azobisisobutyronitrile (AIBN), methyl ethyl ketone (MEK), D-serine, glycidyl methacrylate (GMA), and diethyl ether were purchased from Sigma-Aldrich. Adipic acid dihydrazide (AAD) (97%) was purchased from Alfa Aesar. Poly(glycidyl methacrylate) (PGMA) was synthesized by free radical polymerization according to a previously published procedure [64]. Copper (II) acetate, L-threonine (98%), L-arginine, L-histidine (99%), and L-glutamine were purchased from Fisher Scientific. Ultrapure water (Siemens) (18.2 $M\Omega\text{ cm}$) was used in all experiments. To adjust pH values, 0.1 M aqueous solutions of HCl and NaOH were used. Single-side polished silicon wafers 500- μm -thick (University Wafer) were cut to a typical size of 30 mm x 50 mm and cleaned in a piranha

solution [3:1 (v/v) $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$] for 1 hour. *Attention: Piranha solution is extremely dangerous and should be handled with extreme care!* The slides were thoroughly washed with deionized (DI) water and stored under DI water in a closed container before use.

2.2 Preparation of (PMAA)-EDA multilayer hydrogels

A spin-assisted-LbL method was used to produce hydrogen-bonded films of $(\text{PMAA}/\text{PVPON})_n$ where subscript n denotes the number of adsorbed polymer bilayers. The layer of poly(glycidylmethacrylate) (PGMA) was spin cast from 0.1 mg mL^{-1} chloroform solution on the surface of Si wafers. PGMA was covalently anchored to the surfaces by heating the wafers at 110°C for 1 hour, followed by chloroform rinses to remove uncross-linked PGMA. The first layer of PMAA was deposited from 1 mg mL^{-1} methanol solution and cross-linked at 88°C for 40 min, followed by rinsing with DI water 3 times to remove uncross-linked PMAA. Hydrogen-bonded $(\text{PMAA}/\text{PVPON})_n$ multilayers were deposited on the precursor-treated wafers. Specifically, 0.2 mg mL^{-1} polymer solutions at $\text{pH}=2.5$ were spin-cast onto the precursor-modified Si wafer and rotated at 3000 rpm for 30 s starting from PVPON with M_w 55,000 g mol^{-1} and followed by double rinsing with 0.01 M phosphate solution ($\text{pH}=2.5$). Deposition was repeated until the desired number of bilayers was achieved. After that, the formed hydrogen-bonded multilayers were chemically cross-linked with EDA as described elsewhere [35,65]. Briefly, activation of PMAA carboxylic groups was achieved by immersion of $(\text{PVPON}/\text{PMAA})_{15}$ films into 1 mg mL^{-1} EDC solution at $\text{pH}=5$ (0.01 M phosphate buffer) for 30 min. After activation, the film was transferred into 5 mg mL^{-1} EDA solution at $\text{pH}=5.8$. After cross-linking for 20 hours, PVPON was released from the hydrogel at $\text{pH}=8.5$ overnight. After that, the hydrogel films were deswelled in $\text{pH}=5$ (0.01 M phosphate) buffer and dried under a

mild stream of nitrogen. The dry thickness of the cross-linked hydrogels after removal of PVPON was 26 ± 2 nm, excluding thickness of the precursor.

2.3 Preparation of (PMAA)-AAD hydrogels

The hydrogen-bonded (PMAA/PVPON)_n films were deposited from 1 mg mL^{-1} solution of PMAA and 0.5 mg mL^{-1} of PVPON (M_w 2,500 g mol^{-1}) at pH=2.5 via spin-assisted LBL on PGMA-PMAA-coated wafers as described above. The hydrogen-bonded films were cross-linked with AAD as described above except that EDC solution at pH=4.8 (0.01 M phosphate buffer) was used for activation for 30 min. After activation and rinsing with 0.01 M phosphate buffer at pH=4.6, the film was transferred to a filtered solution of AAD at pH=4.1 (3 mg mL^{-1}) for 3 h. PVPON was released from the hydrogel films in 0.01 M phosphate buffer at pH=8.5 overnight. The dry thickness of the cross-linked hydrogels after removal of PVPON was 28.5 ± 2.0 nm, excluding thickness of the precursor.

2.4 Incorporation of copper (II) ions into (PMAA)-EDA and (PMAA)-AAD

Cross-linked (PMAA)₁₅-EDA and (PMAA)₁₆-AAD hydrogels were exposed to copper (II) acetate at pH=5.8 inside a 5-mL ellipsometer liquid cell (Woollam). Prior to loading, the films were swollen in 0.005 M HEPES buffer at pH=7.5 and immersed in aqueous solution of copper (II) acetate (500 ppm, 4 mL). The wet thickness of copper-loaded film was measured by *in situ* ellipsometry after 10 min. To remove the unbound copper (II) ions, the hydrogel was rinsed 3 times with 0.005 M HEPES at pH=7.5. After each measuring cycle hydrogels were kept in pH=3 (0.01 M) phosphate buffer solution for 10 min to remove the ions before the next ion loading. Stock solutions of amino acids (1000 ppm) were prepared in 0.005 M HEPES buffer and stored at 4°C. A series of dilutions were made from the same stock solution adjusted to

pH=7.5. Copper-loaded hydrogel films were exposed to 4-mL solution of amino acids for 10 min and their thickness was measured. Prior to amino acid loading, 4 mL of buffer (HEPES, 0.005 M, pH=7.5) solution was removed from the cell.

2.5 Ellipsometry

Measurements of the dry film thickness were performed using an M-2000U spectroscopic ellipsometer (Woollam). All dry measurements were done at three angles of incidence: 65°, 70°, and 75°. Liquid cell measurements were carried out in a liquid cell (Woollam) at 75°. To analyze the data, a multilayer model composed of Si, SiO₂ and polymer film was used. The swelling ratio of the hydrogel film is defined as

$$\text{Swelling ratio} = \frac{T_{\text{swollen}}}{T_{\text{dry}}}$$

where T_{dry} is the dry thickness of the hydrogel, not-including precursor, and T_{swollen} is the thickness of the hydrogel in liquid.

2.6 UV-Visible Spectroscopy (UV-vis)

UV-vis spectra of multilayer hydrogel films were recorded using a Cary 100 Bio (Varian). The hydrogen-bonded (PMAA/PVPON)₂₀ films were deposited from 1mg mL⁻¹ buffer solutions (pH=2.5, 5 min deposition time) inside a fluorometry quartz cuvette. Surface adhesion of the subsequently grown multilayer films was enhanced by depositing the first two bilayers of poly(allylamine hydrochloride) (PAH)/PMAA precursors from 1 mg mL⁻¹ aqueous solutions starting from PAH with 15 min per layer adsorption. The precursor layers were cross-linked at 110 °C for 1 hour [66]. The hydrogen-bonded film was cross-linked as described above. Copper (II) and amino acid loading was done as described above for the surface-attached hydrogels. The

absorbance was measured after removing solutions of copper acetate and amino acids and rinsing the cuvette with HEPES. All measurements were done in 0.005 M HEPES at pH=7.5.

2.7 Energy Dispersive Spectroscopy (EDS)

EDS on surface-attached hydrogels was performed using an FEI QuantaTM 650 scanning electron microscope with the EDS capability. For that, the hydrogen-bonded (PMAA/PVPON)₆₀ films were prepared as described above from 1mg mL⁻¹ polymer solutions at pH=2.5. Average precursor thickness was 5.2±0.3 nm with the thickness of the 12-h cross-linked (PMAA)-AAD hydrogels being 95.6±0.7 nm. The size of the Si wafers with the films was 1cm x 1cm. To impregnate hydrogel films with copper (II) ions, 10 mL of 500 ppm aqueous solution of copper (II) acetate was used. Loading time was 10, 30 and 60 min. Each loading was followed by thorough rinsing with 0.005 M HEPES at pH=7.5. For the measurements in the presence of amino acids only the films loaded with copper (II) ions for 10 min were used. For that, the films were immersed in solutions of serine and arginine at different concentrations. After that, the hydrogel films were rinsed with 0.005 M HEPES at pH=7.5 for 10 min and air-dried. As a control, (PMAA)₆₀-AAD hydrogel immersed only in 0.005 M HEPES at pH=7.5 was used.

2.8 *In situ* ATR-FTIR

Chemical cross-linking of (PMAA)_n-AAD and (PMAA)_n-EDA hydrogels as well as copper (II) loading and release in amino acid solutions were monitored with *in situ* Attenuated Total Reflection Fourier Transform Infrared spectroscopy measurements (ATR-FTIR). A Bruker FTIR spectrometer (Vertex 70) was equipped with a narrow-band mercury cadmium telluride detector. The internal compartment of the FTIR spectrometer containing the liquid cell was purged with dry nitrogen. The ATR surface was a rectangular trapezoidal multiple reflection Si

crystal of dimension 50 mm x 10 mm x 2 mm (Harrick Scientific) whose beam entrance and exit surfaces were cut at 45 degrees. Interferograms were collected at 4 cm⁻¹ resolution, and the number of averaged scans was 120. Each interferogram was corrected on the corresponding background, measured for the same ATR cell with the same D₂O buffer solution. The bare ATR crystal was used as a background. To eliminate overlap of the IR bands in the 1700-1500 cm⁻¹ region with the strong water band, D₂O with 99.9% isotope content was utilized. Use of multiple-reflection ATR along with a custom-made flow-through liquid cell allowed *in situ* deposition and compositional monitoring of the ultrathin films (several nanometers in thickness). A 20-bilayer film of (PMAA/PVPON) was deposited using the spin-assisted LbL approach on the surface of the oxidized ATR crystal primed with a PGMA/PEI layer as described for ellipsometry measurements. PGMA was attached to the crystal surface at 70 °C. PEI was deposited from isopropanol and cross-linked at 70 °C for 4 hours. After the film was formed, cross-linking with AAD solutions in D₂O was carried out for 4 hours as described above for silicon wafers. The PVPON was released from the formed hydrogel film by the exposure of the film to 10 mM phosphate buffer at pH=8.5 (D₂O) for 12 hours. For loading Cu²⁺, (PMAA)₁₆-AAD hydrogels were exposed to copper (II) acetate at pH=5.8 in 0.005 M HEPES inside the ATR cell for 20 minutes, followed by rinsing with HEPES buffers at pH=5.8 and pH=7.5. Copper-loaded hydrogel films were then exposed to solutions of amino acids for 10 min and rinsed with HEPES.

2.9 Atomic Force Microscopy (AFM)

AFM images were collected on dry samples using MultiMode 8 (Bruker) microscope in tapping mode. Tips were purchased from Bruker (resonance frequency 300 kHz, force constant 40 N/m, tip radius 10 nm). The images were analyzed using NanoScope Analysis 1.5. For the AFM

analysis, hydrogen-bonded film of (PVPON/PMAA)₁₆/PVPON (PVPON M_w 2500 g mol⁻¹) were cross-linked with EDA and AAD as previously described with the main difference being the pH of EDA (pH=5.5). To impregnate hydrogel films with copper (II) ions, 10 mL of 500 ppm aqueous copper (II) acetate was used. The loading time was 10 min. Each loading was followed by thorough rinsing with 0.005 M HEPES at pH=7.5. For the study of surface morphology after amino acid treatment, the films were immersed in solutions of serine and arginine at 80 ppm followed by rinsing with 0.005 M HEPES at pH=7.5 for 10 min and air-drying.

3. Results and discussion

3.1 Formation of [(PMAA)_n-Cu²⁺] complexes in (PMAA)_n hydrogels

Hydrogel films were prepared by covalent cross-linking of the PMAA carboxylic groups in hydrogen-bonded (PMAA/PVPON)_n multilayers (where n denotes the number of bilayers) as illustrated in Scheme 1a. The hydrogen-bonded films were deposited by a spin-assisted method at pH=2.5 and treated with either AAD or EDA solutions at pH=4.1 and 5.8, respectively, to cross-link the PMAA layers. The films were then exposed to pH=8.5 (0.01 M buffer) to release PVPON by disruption of the hydrogen bonds between PVPON and PMAA, a procedure established earlier for PMAA multilayer hydrogels assembled by dipping [35]. As a result, the film thicknesses decreased after cross-linking to 70% and 65% of their initial thicknesses for (PMAA)₁₆-AAD and (PMAA)₁₅-EDA, respectively. The thickness changed from 38 to 26 nm for (PMAA)₁₆-AAD and from 38 to 24 nm for (PMAA)₁₅-EDA before and after cross-linking as measured with ellipsometry.

Successful formation of a (PMAA)₁₆-AAD hydrogel was confirmed with *in situ* ATR-FTIR. The FTIR spectrum of a hydrogen-bonded film demonstrates two major peaks centered at 1700 cm⁻¹

and 1647 cm^{-1} corresponding to carbonyl stretching vibrations of protonated carboxylic groups (COOH) and carbonyl groups of the pyrrolidone ring (Figure 1a, spectrum 1), respectively [35,45,67]. The spectrum after cross-linking shows a new broad band at 1670 cm^{-1} associated with amide I vibrations and the disappearance of the absorption band associated with PVPON (1647 cm^{-1}) (Figure 1a, spectrum 2). The ionization of not cross-linked PMAA carboxylic groups increases (pK_a of PMAA ~ 6), which is evidenced by the band intensity centered at 1550 cm^{-1} (Figure 1a, spectrum 2). The hydrogel exhibited reversible ionization and protonation at $\text{pH}=8$ and $\text{pH}=3$, respectively, with no PMAA mass loss observed upon pH variations indicating a complete retention of PMAA.

Scheme 1. Schematic representation of (a) PMAA-EDA and (b) PMAA-AAD hydrogels at different pH values.

Scheme 2. Schematic representation of (a) initially swollen PMAA-AAD hydrogels after (b) forming $[PMAA-AAD-Cu^{2+}]$ complexes upon interaction of the hydrogel with a solution of a copper salt, and after their interaction with (c) serine, threonine, glutamine, histidine and (d) arginine solutions at pH=7.5.

Figure 1. (a) FTIR spectra of a (PMAA/PVPON)₂₀ film as deposited at pH=2.5 (spectrum 1), and after cross-linking into (PMAA)₂₀-AAD and exposure to pH=8.5 (spectrum 2). Major absorption bands associated with protonated carboxylic groups (-COOH), ionized carboxylic groups (-COO⁻), and carbonyl vibration of PVPON are centered at 1700, 1550, and 1650 cm^{-1} , respectively. (b) The (PMAA)₂₀-AAD film before (spectrum 1), and after loading with Cu²⁺ at pH=5.8 (spectrum 2). (c) The (PMAA)₂₀-AAD-Cu²⁺ film after treatment with 80 ppm arginine (spectrum 1) or serine (spectrum 2) solutions at pH=7.5. The dashed curve (spectrum 3) shows the copper-free (PMAA)₂₀-AAD film treated with 80 ppm arginine and serine. All spectra are monitored by *in situ* ATR-FTIR in D₂O solutions.

The produced ultrathin multilayer nanostructured hydrogels swell at high pH values due to enhanced ionization of carboxylic acid groups in PMAA chain segments and the inclusion of counter-ions, resulting in Donnan osmotic swelling of the hydrogels. In the case of PMAA-EDA hydrogels, there are three swelling/ deswelling regions (Scheme 1a) [40,65]. The swelling from pH=6 to pH=8 is explained by increased ionization of PMAA carboxylic groups (pK_a of PMAA is ~ 6). The swelling at $pH < 5$ and $pH > 9$ originates from primary amino groups of the single-end reacted cross-linker molecules supplied from the EDA cross-linker. The both-end reacted EDA molecules provide permanent covalent cross-links within the PMAA-EDA hydrogel. The single-end attached EDA molecules also afford additional ionic cross-linking of the hydrogel through ionic pairing between $-\text{COO}^-$ groups of PMAA network and $-\text{NH}_3^+$ groups of single-end attached EDA molecules. The additional ionic cross-linking is the reason for the lowest swelling degree of the PMAA-EDA hydrogel observed at pH=5 and corresponding to the hydrogel most compact structure where both types of hydrogel cross-links are present. In contrast, when the PMAA hydrogel is cross-linked with a more hydrophobic AAD cross-linker, there is no hydrogel swelling at low pH (Scheme 1b), which is because of the higher reactivity and a longer length of AAD allowing both ends of the cross-linker to participate in the reaction with carboxylic groups as has been reported previously for dipped PMAA hydrogels [55].

The AFM analysis of $(\text{PVPON/PMAA})_{16}$ films reveals a moderate increase in the surface microroughness (RMS) from 1.24 ± 0.04 to 1.45 ± 0.10 nm with the increase in pH from 2 to 5, respectively (Table 1). The elevated surface roughness can be explained by the PVPON chain rearrangements due to the partially ionized PMAA, occurring similar to the previous study on hydrogen-bonded films [68]. However, the film morphology was significantly smoothened after cross-linking (Figure 2). The RMS of hydrogels decreased from 1.45 ± 0.10 to 0.48 ± 0.03 nm and

to 0.76 ± 0.03 nm for (PMAA)₁₆-EDA and (PMAA)₁₆-AAD, respectively, when measured at pH=5. The smooth morphology of (PMAA)_n hydrogels was previously explained by the relaxation of cross-linked PMAA chains due to PVPON release [69]. A twice higher RMS of (PMAA)₁₆-AAD hydrogels as compared to that of (PMAA)₁₆-EDA can be attributed to the higher hydrophobicity of the AAD cross-linker due to the presence of additional methylene groups in its chemical structure. A significant decrease in surface roughness to 0.33 ± 0.01 and 0.36 ± 0.02 nm for (PMAA)₁₆-EDA and (PMAA)₁₆-AAD systems, respectively, was observed after exposure of these hydrogels to pH=8 to complete PVPON removal. The result is consistent with previously reported smoothening of (PMAA)₂₀-EDA hydrogels at pH=8 [69]. In our case, similar morphology of both hydrogels indicates a predominant effect of chain extensions due to the electrostatic repulsions in the ionized state and thus a more hydrophilic PMAA network independent of the cross-linker type.

Hydrogel films were then impregnated with copper (II) ions and subsequently exposed to a series of amino acid solutions (Scheme 2a-b). Histidine, glutamine, serine, arginine and threonine were chosen due to the presence of -CONH₂, -OH, imidazole and guanidine functional groups available to interact with copper (II) ions (Scheme 3) [70,71]. Figure 3 shows the pH-dependent swelling ratio profiles of both types of hydrogels before and after loading with copper (II) ions as measured with *in situ* ellipsometry. One can see that prior to Cu²⁺ loading, (PMAA)₁₅-EDA showed amphoteric swelling (pH<5 and pH>5) due to ionization of carboxylic (pK_a of PMAA ~6) and amino groups (pK_a of -NH₂ is ~10) in the cross-linked network [35]. In contrast, the (PMAA)₁₆-AAD film did not show an increase in thickness at pH<5, but started to swell gradually at pH≥5. The different swelling behavior was explained by the low pK_a of hydrazide groups ($pK_a=2.5$) in comparison with that of amino groups ($pK_a \sim 10$) [55,72]. The swelling ratios

for (PMAA)₁₆-AAD and (PMAA)₁₅-EDA at pH=7.5 were 7.4 and 9.5, respectively (Figure 3b). The pH-dependent swelling profiles of both types of the spin-assisted hydrogels correlate well with the previously reported data for PMAA hydrogels obtained by dipping [35,40,50,55,65]. The lower swelling of (PMAA)₁₆-AAD compared to (PMAA)₁₅-EDA was explained by the higher hydrophobicity of the AAD cross-linker [55,66]. However, the spin-assisted systems studied here showed much greater swelling compared to the conventional ‘dipped’ hydrogels found to fall in a range from 3 to 5 [35,55]. In contrast, hydrogen-bonded films before cross-linking swell only 1.2 fold their dry thickness in the pH range from 2.5 to 5.5 until their total disintegration at pH=5.8. (Figure S2). The result indicates that the pH-triggered swelling is suppressed in the presence of PVPON, despite partial ionization of PMAA groups (~14% [73]).

Loading these hydrogels with copper (II) ions results in a significant decrease in swelling from 9.5 to 3 and from 7.4 to 3.3 times the dry thickness for (PMAA)₁₅-EDA and (PMAA)₁₆-AAD, respectively (Figure 3b). Suppressed swelling of the hydrogels at pH=7.5 implies that the Cu²⁺ interacts with the carboxylic groups of PMAA and constrains chain rearrangements. This result agrees well with our previous data on decreased swelling of PMAA capsules in the presence of Mn²⁺ ions [41]. In that work, pH-sensitivity of PMAA hydrogel capsules cross-linked with EDA was constrained by the Mn²⁺ ions produced during MnCO₃ core dissolution. Removal of the ions from the capsule wall with ethylenediaminetetraacetic acid (EDTA) restored the capsule pH-dependent behavior [41]. Similarly, divalent metal ions induced shrinkage of supramolecular hydrogels containing carboxylic groups, bulk poly(methacrylic acid) gels and brushes [74-76]. We found that Cu²⁺ could be completely removed from the (PMAA)_n-EDA and (PMAA)_n-AAD hydrogels at pH=3 leading to total recovery of hydrogel swelling (Figure 3c). The Cu²⁺ release from the [PMAA-EDA-Cu²⁺] and [PMAA-AAD-Cu²⁺] hydrogel complexes at low pH is due to

the accumulation of excess positive charge from ammonium groups in the case of PMAA-EDA hydrogels, and mutual repulsion of Cu^{2+} cations from PMAA-AAD hydrogels upon protonation of ionized carboxylic groups [40, 66]. Cu^{2+} could be also released from multilayer films of weak polyelectrolytes using hydrochloric acid as was shown previously [61].

Table 1. Summary of the AFM data analysis of the hydrogen-bonded $(\text{PVPON } 2.5\text{K}/\text{PMAA})_{16}$ PVPON films and hydrogels cross-linked with ethylene diamine and adipic acid dihydrazide.

pH/LBL assembly	$(\text{PVPON}/\text{PMAA})_{16}\text{PVPON}$	$(\text{PMAA})_{16}\text{-EDA}$	$(\text{PMAA})_{16}\text{-AAD}$
	Root mean square microroughness (RMS), nm		
pH=2.5	1.24 ± 0.04	n/a	n/a
pH=3	n/a	0.43 ± 0.01	0.77 ± 0.06
pH=5	1.45 ± 0.10	0.48 ± 0.03	0.76 ± 0.03
pH=8	n/a	0.33 ± 0.00	0.36 ± 0.02

To investigate the effect of time on loading capacity, $(\text{PMAA})_{60}\text{-AAD}$ hydrogels were exposed to Cu^{2+} solutions for 10, 30 and 60 min followed by rinsing with HEPES buffer at pH=7.5 to remove unbound copper (II) ions. The presence of Cu^{2+} ions in the hydrogels was monitored with Energy Dispersive Spectroscopy (EDS) (Figure 4a). The peaks at 0.93 and 8.04 keV corresponding to copper (II) ions showed similar intensity after 10, 30 and 60 min of loading. This result indicates that 10 min is sufficient to saturate the hydrogel with Cu^{2+} ions (Figure 4b,c).

Figure 2. AFM images of hydrogen-bonded (PMAA/PVPON)₁₆ film assembled at pH=2.5 (a,b). Cross-linked (PMAA)₁₆-AAD hydrogels dried from pH=5 (c, d) and pH=8 (j, k). AFM images of cross-linked (PMAA)₁₆-EDA hydrogels dried from pH=5 (e, f) and pH=8 (l, m). The Z- height is 15 nm.

The ATR-FTIR spectra of (PMAA)₂₀-AAD hydrogel before and after exposure to the Cu²⁺ at pH=5.6 are shown in Figure 1b. The spectra show that ionization of PMAA significantly increased after complexation with copper ions, which is evidenced by the decreased band intensity centered at 1700 cm⁻¹ but the increased peak at 1550 cm⁻¹ associated with protonated and ionized carboxylic groups, respectively. No changes in the spectrum were observed after rinsing with pH=8.5. Another important observation is that the band at 1550 cm⁻¹ is broader as compared to that of the ionized PMAA at pH=8.5 (Figure 1a). As shown previously, complexation of carboxylic groups with copper (II) ions involves interactions of two carboxylate moieties with one or two central Cu²⁺ to form chelating and bridging bidentate complexes, respectively [60,77,78]. Konradi and Rhe have studied PMAA brush-copper (II) coordination and assigned two bands centered at 1558 cm⁻¹ and at 1611 cm⁻¹ for chelating bidentate and stronger bridging bidentate complexes, respectively, with the bridging bidentate complexation

explained to be stronger and more favorable [60]. In our case, peak broadening along with the appearance of a shoulder centered at 1610 cm^{-1} indicate that Cu^{2+} forms complexes with $(\text{PMAA})_n$ hydrogels through both chelating bidentate and bridging bidentate coordination. The copper-hydrogel complexation enhances PMAA ionization, the phenomenon found earlier for PMAA brushes that showed induced PMAA ionization due to metal binding and in ionically-paired multilayers when topped with a polycation [58,60].

Scheme 3. Structure of threonine, serine, histidine, glutamine and arginine.

AFM analysis of surface morphology revealed a significant increase in microroughness from 0.34 ± 0.01 to 1.32 ± 0.01 nm before and after copper treatment, respectively, which is an additional indication of hydrogel-copper complexation (Figure 5, Table 2).

Our aforementioned data show that both $(\text{PMAA})_n$ -AAD and $(\text{PMAA})_n$ -EDA hydrogels can efficiently load copper (II) ions. Complexation of Cu^{2+} with hydrogels results in suppressed hydrogel swelling, elevated PMAA ionization, and increased surface roughness. The mechanism of copper-hydrogel interactions is pH-dependent and complex due to the ampholytic nature of

(PMAA)_n networks containing various types of functional groups. We suggest that Cu²⁺ forms two types of interactions at neutral pH including binding to ionized carboxylic groups and to amine or hydrazide groups provided by the cross-linkers. A similar mechanism was suggested for PEI/PAA multilayers impregnated with copper ions [56,58]. Release of Cu²⁺ from the hydrogels at low pH can be explained by full protonation of carboxylic groups causing diminished interactions between Cu²⁺ and PMAA. Also, partial protonation of amino groups at low pH favors repulsion of positively charged Cu²⁺ ions.

3.2 Stability of (PMAA)_n-Cu²⁺ complexes in amino acid solutions

The stability of (PMAA)_n-Cu²⁺ interactions in the presence of amino acids was first investigated by following swelling behavior of both types of networks with *in situ* ellipsometry. The Cu²⁺ loaded hydrogels were immersed in amino acid solutions of 8 and 80 ppm for 10 minutes and rinsed with buffer at pH=7.5 (Figure 6). As seen in Figure 6, [(PMAA)₁₅-EDA-Cu²⁺] shows a two-fold increase in the swelling ratio to 5.3 and 5.6 in the presence of 8 ppm threonine and serine, respectively, as compared to amino acid-free buffer. No changes in film thickness were observed in histidine, arginine and glutamine under those conditions (Figure 6a). Increasing the concentration of amino acids to 80 ppm lead to increased swelling ratios of the hydrogel up to 9, which was similar to Cu²⁺-free hydrogels. The result indicates that total recovery of [(PMAA)₁₅-EDA-Cu²⁺] swelling is independent of the amino acid concentration (Figure 6a). In contrast, 8 ppm amino acids did not affect [(PMAA)₁₆-AAD-Cu²⁺] swelling which remained suppressed when treated with all five amino acids (Figure 6b). Moreover, its response to higher amino acid concentration was also different as compared to the [(PMAA)₁₅-EDA-Cu²⁺] hydrogel. The [(PMAA)₁₆-AAD-Cu²⁺] system showed a swelling ratio of 6.1 in serine and glutamine, and 6.7

in histidine and threonine, indicating a partial recovery of swelling behavior in 80 ppm solutions. No change in the swelling ratio was observed in the presence of arginine at those concentrations.

Figure 3. (a) *In situ* ellipsometry study of (PMAA)₁₆-AAD (squares) and (PMAA)₁₅-EDA (triangles) hydrogel swelling as a function of pH. The dry thicknesses of (PMAA)₁₆-AAD and (PMAA)₁₅-EDA were 26.4 and 23.9 nm, respectively, excluding thickness of the precursor; (b) Swelling ratio of the (PMAA)₁₅-EDA and (PMAA)₁₆-AAD films loaded with copper (II) ions; (c) Summary of the steps taken to reversibly load/unload PMAA-based hydrogels: 1) loading, hydrogel swells at pH=8; 2) absorption of Cu²⁺ in the hydrogel; 3) suppressed swelling at pH=7.5 (0.005 M HEPES); 4) removing Cu²⁺ from the hydrogel by exposure of the hydrogel to pH=3; 5) recovery of the swelling at pH=8;

Figure 4. EDS data for comparing the effect of time on the amount of copper (II) ions absorbed by the (PMAA)₆₀-AAD hydrogels. The hydrogel was soaked in copper (II) solutions for 10, 30 and 60 minutes followed by rinsing with 0.005 M HEPES at pH=7.5. The entire spectrum of the hydrogel (a); zoom in to the regions corresponded to Cu (II) absorbance area at 0.93keV (b) and 8.04keV (c).

Figure 5. A cross-linked (PMAA)₁₆-AAD hydrogels dried from HEPES at pH=7.5 (a,b); (PMAA)₁₆-AAD-Cu²⁺ followed by washing with HEPES at pH=7.5 (c,d); (PMAA)₁₆-AAD-Cu²⁺-arginine at 80 ppm followed by washing with HEPES at pH=7.5 (e, f); (PMAA)₁₆-AAD-Cu²⁺-serine at 80 ppm followed by washing with HEPES at pH=7.5 (g, h). The Z- height is 15 nm.

Table 2. Summary of the AFM data analysis of (PMAA)₁₆-AAD hydrogels exposed to copper (II) ions, buffer and 80 ppm serine and arginine solutions

	HEPES, pH=7.5	Cu ²⁺ -HEPES, pH=7.5	Cu ²⁺ - 80 ppm arginine-HEPES, pH=7.5	Cu ²⁺ - 80 ppm serine-HEPES, pH=7.5
RMS, nm	0.34±0.01	1.32±0.01	1.16±0.04	0.5±0.01

Thus, amino acids remove copper (II) ions from hydrogels and recover network swelling to the degree controlled by the amino acid type and concentration (Scheme 2c). Partial removal of Cu (II) ions from [(PMAA)₁₅-EDA-Cu²⁺] complex in 8 ppm amino acid solutions indicates that binding of Cu (II) ions to (PMAA)₁₅-EDA is weaker than to (PMAA)₁₆-AAD. At the same time, [(PMAA)₁₆-AAD-Cu²⁺] showed selective trapping and recognition of arginine among other amino acids in 80 ppm solutions (Scheme 2d). These variations in hydrogel swelling are controlled by the amount of copper (II) ions remained in the hydrogels.

To investigate the degree of the copper (II) ion removal, (PMAA)_n-AAD hydrogels were tested with UV-vis spectroscopy, EDS, and *in situ* ATR-FTIR. The UV-vis revealed that the absorption spectrum of a copper-free (PMAA)₂₀-AAD was transparent between 230 and 340 nm (Figure 7). The absorption band appeared at 260 nm after soaking this hydrogel in the solution of copper acetate indicating interaction of Cu²⁺ with the hydrogel matrix. The 260-nm absorption band was also found in (BPEI/PAA) films in copper (II) nitrate solutions and attributed to Cu²⁺-(BPEI/PAA)₁₀ complex formation [61]. However, the peak intensity at 260 nm significantly decreased after exposure of the copper-loaded [(PMAA)₂₀-AAD-Cu²⁺] to an 80 ppm solution of serine, indicating copper ion removal (Figure 7a). In contrast, a small change in the band intensity was observed in arginine solution under similar conditions, confirming retention of Cu²⁺ in the film (Figure 7b). EDS confirmed selective removal of Cu²⁺ ions from [(PMAA)₆₀-AAD-Cu²⁺] films in serine solutions (Figure 8b). The EDS spectrum of a copper-loaded [(PMAA)₆₀-AAD-Cu²⁺] witnessed the presence of characteristic Cu peak at 0.93 keV. No peak was present in this region for a copper-free (PMAA)₆₀-AAD hydrogel. The peak at 0.93 keV was significantly decreased upon hydrogel exposure to 80 ppm serine. In contrast, only a small decrease in the band intensity was observed in 80 ppm arginine solution.

Figure 6. (a) Swelling ratio of the (PMAA)₁₅-EDA film in the presence of different amino acids at 8 ppm and 80 ppm. (b) Swelling ratio of (PMAA)₁₆-AAD in the presence of amino acids at 8 ppm and 80 ppm. The dashed line shows the hydrogel swelling ratio at pH=7.5 when the Cu (II) ions were not present. (Equilibration time before the measurements was 10 minutes).

Figure 7. UV-vis absorbance spectra of a (PMAA)₂₀-AAD film before (black line) and after (red line) exposure to copper (II) ions followed by addition of serine (a) and arginine (b) at 80 ppm (blue lines). The hydrogel was exposed to Cu²⁺ and amino acid solutions followed by rinsing with 0.005 M HEPES at pH=7.5. Inset shows the spectra of the hydrogel in the presence of serine (a) and arginine (b) solutions at 80 ppm.

Figure 8. EDS spectra of the (PMAA)₆₀-AAD films after soaking in copper (II) and exposure to serine and arginine at 80 ppm. The hydrogels were washed with 0.005 M HEPES at pH=7.5 prior the measurements. (a) The entire spectra of the hydrogels; (b) zoom in to the regions corresponding to Cu (II) absorbance area at 0.93 keV. Red line-(PMAA)₆₀-AAD); Blue line-hydrogel loaded with Cu (II); Green line- hydrogel loaded with copper (II) and exposed to arginine at 80 ppm; Pink line-hydrogel loaded with copper (II) and exposed to serine at 80 ppm.

To explore the effect of amino acid concentration on the removal of Cu (II) ions, [(PMAA)₁₆-AAD-Cu²⁺] was treated with arginine solutions > 80 ppm. We found that swelling of the [(PMAA)₁₆-AAD-Cu²⁺] hydrogel was suppressed in the presence of arginine at low

concentration (≤ 80 ppm) followed by a gradual increase in the swelling ratio above 800 ppm (Figure 9a). The swelling ratio of the hydrogel-copper complex increased from 3.1 to 5.5 with an increase in arginine concentration from 80 ppm to 960 ppm, respectively (Figure 9a). The ellipsometry data revealed that the hydrogel swelling ratio reached a plateau of ~ 5.5 at arginine concentration above 800 ppm. The enhanced swelling correlated well with partial removal of Cu (II) ions observed with EDS and UV-vis. EDS showed a decrease in the copper peak intensity with an increase in arginine concentration from 80 ppm to 400 ppm (Figure 10a,b). Similarly, a 260-nm absorbance peak associated with Cu^{2+} consistently decreased upon exposure of copper-loaded [(PMAA)₂₀-AAD-Cu²⁺] hydrogels to 200, 750 and 1000 ppm arginine solutions (Figure 9b). At the same time, UV-vis data indicated that increasing the concentration of arginine above 750 ppm did not completely take away copper ions from the hydrogel. The concentration effect found here agrees well with previous data on the change in fluorescence intensity of polymer-copper (II) complexes in the presence of amino acids [22]. In that work, recovery/quenching changed linearly only in the short range of the amino acid concentrations ($7\text{-}30\ \mu\text{M mL}^{-1}$ in the presence of glycine) followed by reaching a plateau.

ATR-FTIR spectra of [(PMAA)₂₀-AAD-Cu²⁺] hydrogels exposed to arginine and serine at 80 ppm followed by washing with pH=7.5 buffer are shown in Figure 1c (spectrum 1 and 2, respectively). The spectrum after arginine treatment shows two additional bands centered at $1608\ \text{cm}^{-1}$ and at $1590\ \text{cm}^{-1}$ associated with -NH- bending and -COO^- vibrations in proteins [79,80], which confirms arginine presence in the hydrogel. In contrast, no protein peaks are observed in the spectrum after exposure to serine. A small distinct band at $1610\ \text{cm}^{-1}$ in this spectrum originates from the bridging bidentate copper (II) complexes that remain coordinated with the hydrogel. This peak was also observed in the (PMAA) hydrogel after Cu (II) loading (Figure 1b,

spectrum 2) The presence of bridging bidentate instead of chelating bidentate coordination is in good agreement with the previously reported stronger complexation via two central metal ions [60]. The thickness of the vibration band at 1550 cm^{-1} in this spectrum is similar to that in a Cu (II)-free spectrum (Figure 1c, spectrum 3) that indicates that most of the copper (II) ions are removed by serine. Importantly, there are no interactions between the copper (II)-free hydrogel with either serine or arginine at pH=7.5 (Figure 1c, spectrum 3). This result along with the fact that no decreased or increased swelling was observed for the copper-free hydrogel in the presence of amino acids at pH=7.5 and at pH=5.8, respectively, as measured by *in situ* ellipsometry (Figure SI 3) is evidence that the interaction of (PMAA)₂₀-AAD hydrogels with arginine is only possible through coordination with copper (II) ions.

The FTIR data are in good agreement with the AFM results that revealed smoothening of the hydrogel surfaces after exposure to 80 ppm serine solutions (Figure 5g,h) with the microroughness decreasing from 1.32 to 0.5 nm (Table 2). In contrast, only a small change in the microroughness from 1.32 to 1.16 nm was observed after treatment with 80 ppm arginine indicating that a significantly higher amount of copper (II) remained in the hydrogel.

To test if the selective removal of Cu (II) ions by arginine is due to the limited diffusion of amino acids, the mesh sizes of both types of hydrogels were analyzed. We have previously found that the (PMAA)₁₅-EDA hydrogel contains 6.5 monomer units between two cross-links and corresponding mesh sizes of 2.7 nm at pH=5 [50]. The calculated cross-link density of the (PMAA)₁₆-AAD hydrogels studied here is 12.6 monomer units between two cross-links giving mesh size of 3.8 nm at pH=3 (the most compact state of the hydrogel). The hydrodynamic sizes of serine, threonine, arginine, histidine and glutamine were shown to be in the range of 0.25 – 0.4 nm [81]. Apparently, the small size of amino acids allows their fast and unrestrained penetration

into the (PMAA)₁₅-EDA and (PMAA)₁₆-AAD hydrogels. Thus, the degree of copper (II) removal from these networks is mostly controlled by Cu²⁺ interactions with the hydrogel and the amino acid, similar to ligand exchange in copper (II) complexes with proteins and small organic molecules or amino acids in solutions [82,83].

Figure 9. *In situ* ellipsometry study of the swelling of (PMAA)₁₆-AAD hydrogels in the presence of arginine at different concentrations (a). Absorbance spectrum of (PMAA)₂₀-AAD loaded with Cu²⁺ (blue line) after interaction with arginine at 200 ppm (red line), 750 ppm (wine line) and 1000 ppm (dark yellow line) (b). The black line represents the spectrum of (PMAA)₂₀-AAD film without presence of copper (II).

Figure 10. EDS spectra comparing the amount of copper (II) ions in the (PMAA)₆₀-AAD hydrogels after interaction with arginine at 80 ppm and 400 ppm. The hydrogels were washed with 0.005 M HEPES at pH=7.5 prior to the measurements. (a) The entire spectrum of the hydrogel; (b) zoom in to the regions corresponding to Cu (II) absorbance area at 0.93 keV .

Our overall results show that the stability of [hydrogel-Cu²⁺] complexes in amino acid solutions are mainly controlled by the strength of two types of interactions including [(PMAA)-Cu²⁺] bonding and the interaction between Cu²⁺ and amino acids. It is generally accepted that amino acids interact with copper (II) ions via carboxylic and amino groups [17,18]. Stability constants of binary Cu-(arginine)₂, Cu-(glutamine)₂, Cu-(histidine)₂, Cu-(serine)₂ and Cu-(threonine)₂ complexes in 0.15 M KNO₃ were found to be of 4.6x10¹³, 2.5x10¹³, 3.2x10¹⁷, 1x10¹⁴ and 1x10¹⁴, respectively [84]. The reported values indicate the following order of stability for Cu (II) binary complexes: glutamine≤arginine<serine=threonine< histidine. Similarly, dissociation constants of binary Cu-(arginine)₂, Cu-(serine)₂ and Cu-(threonine)₂ were 18x10¹⁵, 2.9x10¹⁵ and 2.9x10¹⁵, respectively and were measured in 0.06 M KH₂PO₄ [89]. The higher value of dissociation constant for Cu-(arginine)₂ indicated lower stability of this complex as compared to serine- and threonine- complexes and was explained by the close proximity of positively charged (=NH₂⁺) groups unfavourable for binding with copper (II) ions. Importantly, the stability constants of binary amino acids-copper (II) complexes are significantly higher than those reported for PMAA-copper (II) bulk gels found to be of 2x10⁻⁵ and 3.98 x10⁻⁶ for 1% and 9% of cross-linker in the gel [85].

The reported constants suggest that the interaction between Cu²⁺ and amino acids are much stronger than that of Cu²⁺ and PMAA which should result in competition of amino acids with the PMAA hydrogel for the ions following by fast expulsion of [amino acid-Cu²⁺] complexes from the network matrix. This phenomenon is observed with our hydrogels when amino acid concentrations are above 800 ppm. At lower concentration (≤ 80 ppm) the degree of copper (II) removal is strongly controlled by the amino acid and the cross-linker type. This effect is clearly

observed with [(PMAA)₁₆-AAD-Cu²⁺] hydrogel that entraps arginine from 80 ppm solutions unlike [(PMAA)₁₅-EAD-Cu²⁺] which loses Cu²⁺ to arginine under these conditions.

The special affinity of [(PMAA)₁₆-AAD-Cu²⁺] hydrogels to arginine among other amino acids is due to the high stability of ternary (hydrogel-Cu²⁺-arginine) complexes (Scheme 4). It was previously shown that addition of amino acids to the [Cu²⁺-PMAA] bulk hydrogels in solutions resulted in binary Cu-(amino acid)₂ and ternary Cu-(amino acid)-(PMAA) mixed ligand complexes at pH>7 with a higher probability of ternary complex [70]. The higher population of ternary mixed ligand complexes was attributed to a statistical effect and increased stability of mixed complexes due to intermolecular interactions between groups not participating in metal binding [71,86]. For our systems, the stability of [hydrogel-Cu²⁺-arginine] complexes can be explained by several factors. First, Cu²⁺-arginine interactions are relatively weak and unfavorable to remove copper (II) ions from the PMAA hydrogels. Our ellipsometry data show that the efficiency of Cu²⁺ removal and the corresponding strength of Cu²⁺-amino acid binding increases in the following order: arginine < glutamine = histidine < serine=threonine, which correlates well with the aforementioned values of stability constants for binary Cu-(amino acid)₂ complexes. Second, guanidine side groups favor [PMAA-arginine] interactions (Scheme 4b,c). For example, Yamauchi *et al.* reported that stability constants of [arginine-Cu²⁺-ethylenediamine-N-monoacetic acid] complexes were improved by 41% as compared to those with alanine [70]. Indeed, among the selected five amino acids, threonine, serine, and glutamine have a negative net-charge at pH=7.5 with their isoelectric points of *pI*=5.6, *pI*=5.68, and *pI*=5.65, respectively. Only arginine and histidine with the corresponding *pI* values of 10.76 and 7.59 have positively charged groups on the side chain (Scheme 3) [87-89]. In our case, additional ionic pairing and hydrogen bonds between carboxylic groups of PMAA and the guanidine side group of arginine

additionally stabilize [hydrogel-copper-arginine] complexes but not [hydrogel-copper-histidine] (Scheme 4b,c) similar to ternary Cu^{2+} -mixed-ligand complexes [90]. Finally, another very important parameter is associated with a stronger binding of Cu (II) ions to $(\text{PMAA})_{16}$ -AAD as compared to $(\text{PMAA})_{15}$ -EDA due to the more hydrophobic nature of AAD [55,66].

Scheme 4. Suggested interactions between Cu^{2+} ions, positively charged guanidinium-groups of arginine and negatively charged carboxylic groups of $(\text{PMAA})_n$ -AAD multilayer hydrogels at pH=7.5. For simplicity, guanidinium-side groups of arginine are presented as NH_2^+

Finally, we found that the (PMAA)₁₆-AAD hydrogel could be recycled multiple times. Copper (II) ions and arginine remaining in the hydrogel were completely removed at pH=3 fully recovering network swelling. Because of this, copper and amino acid loading and release could be repeated multiple times with the same hydrogel (Figure 3c), which is an important property for applying these systems in sensing and actuation.

4. Conclusions

We demonstrated the ability of (PMAA)-based nanothin hydrogel films to form complexes with copper (II) ions at neutral pH as a result of Cu²⁺ binding to ionized carboxylic groups and to amine or hydrazide groups provided by the cross-linkers. We showed that the stability of these [(PMAA)-Cu²⁺] complexes in amino acid solutions was strongly controlled by the amino acid type, concentration and the cross-linker used in hydrogel synthesis. We found the swelling of the hydrogels to be significantly suppressed after loading copper (II) ions but restored by treatment with amino acid solutions including serine, threonine, arginine, histidine and glutamine. The swelling recovery was controlled by Cu²⁺ removal from the hydrogel which was regulated by the strength of [(PMAA)-Cu²⁺] bonding and by [Cu²⁺-amino acid] interactions. The efficiency of Cu²⁺ removal and the corresponding strength of [Cu²⁺-amino acid] binding increased in the following order: arginine < glutamine = histidine < serine=threonine. We found that the special affinity of [(PMAA)₁₆-AAD-Cu²⁺] hydrogels to arginine is due to the formation of ternary [hydrogel-Cu²⁺-arginine] complexes stabilized by electrostatic interactions between carboxylic groups of PMAA and the guanidinium side group of arginine. No interaction was found between Cu²⁺-free hydrogels and arginine indicating a crucial role of copper (II) ions in ternary complex formation. Considering several unique features including (a) easily-controlled thickness at the nanoscale, (b) high swelling capacity, (c) fast Cu²⁺ loading without chelating agents, (d)

recognition of several types of amino-acids, and (e) multiple detection cycles without altering original hydrogel swelling profile, these hydrogels hold a considerable promise for amino-acid sensing.

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*Graphical Abstract

■ Serine, threonine,
glutamine, histidine

■ Arginine

pH = 7.5

Cu^{2+}

pH = 7.5