poly-HEMA/TMPT ハイドロゲルを用いた抗菌剤の長期徐放システムの開発

Development of sustained antimicrobial-release systems using poly-HEMA/TMPT hydrogels

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GENERAL INTRODUCTION

Reconstructive materials with antimicrobial effects could be useful in medical or dental fields for preventing infectious diseases in an environment containing indigenous bacteria or fungi. A conventional approach to providing reconstructive materials with infection control abilities is to incorporate water-soluble antimicrobials and enable their release in a wet environment. For example, to prevent bacterial infections associated with orthopaedic implants or intravascular catheters, surface treatmennt of these materials with antimicrobials such as biguanides or quaternary ammonium compounds have been attempted [1, 2]. In dental field, several studies on addition of antimicrobials, such as cetylpyridinium chloride, benzalkonium chloride, or chlorhexidine, to composite resins [3], glass-ionomer cements [4-7], orthodontic adhesives [8, 9], endodontic filling materials [10], or acrylic varnishes [11] are available. However, the antimicrobial effects displayed by these materials are limited to a short period and continuous delivery of the agents is not possible. Such limitation is crucial in an oral environment harboring plenty of bacteria and fungi, and dental reconstructive materials with sustained antimicrobial effects are necessary to effectively prevent bacterial/fungal infectious diseases.

One approach to solving this problem is to immobilize an antimicrobial component in restorative materials by incorporation of a polymerizable bactericide such as quaternary ammonium compound (QAC)-based resin monomers, represented by 12-methacryloyloxydodecylpyridinium bromide (MDPB) [12-15]. MDPB has been utilized for various resin-based materials, such as composite resins [12, 16, 17], pre-polymerized resin fillers [18, 19], or adhesives [14]. Besides MDPB, several other

studies reported application of newly developed QAC-based monomers to adhesives or composite resins [20-22]. Antimicrobials immobilized by polymerization of these QAC-based monomers do not leach out from their carrier materials and long-lasting effects can be obtained. However, since materials with immobilized bactericides exhibit antimicrobial effects that are dependent upon contact inhibition of bacteria, their effectiveness can be readily reduced by coverage with protein.

A potential new approach to providing reconstructive materials with long-lasting antimicrobial effects is to apply non-biodegradable carriers for antimicrobials as drug reservoirs. For this, non-biodegradable hydrogels, such as poly-2-hydroxyethyl methacrylate (poly-HEMA), may be useful because they can effectively take up water-soluble antimicrobials in conjunction with water and release them in a wet environment [23-26]. In addition, exposure of such hydrogels to antimicrobial solutions may enable recharging with antimicrobials, thus achieving persistent antimicrobial effects.

OBJECTIVES AND CONTENTS

With the objective of applying a non-biodegradable hydrogel to resin-based materials as a reservoir for water-soluble antimicrobials, novel hydrogels consisting of HEMA and trimethylolpropane trimethacrylate (TMPT) were fabricated, and cetylpyridinium chloride (CPC), a type of QAC, was loaded into these hydrogels.

The general objective of this thesis work was to develop sustained antimicrobial-release systems using CPC-loaded poly-HEMA/TMPT hydrogels by evaluating their ability to release and recharge CPC *in vitro*.

In chapter 1, five poly-HEMA/TMPT hydrogels comprising different ratios of HEMA/TMPT were fabricated and loaded with CPC by immersion into a CPC solution. Then, the ability of these five hydrogels to release CPC was evaluated. In chapter 2, the concentration of CPC required to inhibit oral bacteria and fungi was determined for designing recharge protocol of CPC, and the ability of poly-HEMA/TMPT hydrogels to recharge CPC was evaluated. In addition, poly-HEMA/TMPT hydrogels containing CPC were prepared by pre-mixing of CPC powder into hydrogels, and their release and recharge capacities of CPC were evaluated.

CHAPTER 1

Fabrication of poly-HEMA/TMPT hydrogels and evaluation of their ability to release CPC

1.1 Materials and methods

1.1.1 Fabrication and characterization of poly-HEMA/TMPT hydrogels

For fabrication of hydrogels, HEMA (Tokyo Kasei, Tokyo, Japan, 97.6% purity) and cross-linking monomer, trimethylolpropane trimethacrylate (TMPT; Shin-nakamura Kagaku, Wakayama, Japan, 94.8% purity) were used (Fig. 1). With 0.5 weight (wt) % benzoyl peroxide, mixtures of HEMA and TMPT at weight ratios of 10/90, 30/70, 50/50, 70/30, or 90/10 were heat polymerized first at 120°C for 2 h and then under diminished pressure (−0.1 megapascal) for 16 h (Table 1). The prepared polymer was ground into particles with a diameter of 500 ± 22 μm using a laboratory mill (MF10 basic; IKA, Staufen, Germany) and a planetary ball mill (LP-1; Ito Seisakusho, Tokyo, Japan). The powders were washed by immersion in distilled water for 48 h at 25°C and dried for 24 h at 60°C.

To determine the water absorption capacity of the hydrogels, 1 g of each hydrogel particle was immersed in 25 g of water and stored for 96 h at 25°C, with agitation. After vacuum filtration on filter paper (No.2; Advantec, Tokyo, Japan) for 3 min, the weight of the particles was measured using an electronic balance (PB1502-s/FACT; Mettler-Toledo, Greifensee, Switzerland), and the water absorption ratio was calculated using the following equation:

$$\text{Water absorption ratio (\%)} = (W_b - W_a) / W_a \times 100,$$

where W_a is the weight before storage in water and W_b is the weight after storage in water for 96 h.

To assess hydrophobicity, 100 mg (W_d) of each hydrogel particle was immersed in 1 mL of either distilled water or aqueous 70 vol% isopropanol. After 36 h, the swollen weight (W_s) of the particles was measured. The equilibrium mass swelling ratio (q) in solvent x (I70: 70 vol% isopropanol, H_2O: distilled water) was calculated as: $q_x = W_s/W_d$. The *H*-index of each hydrogel was then determined as: $H = q_{I70}/q_{H_2O}$.

To determine the surface electric charge, each hydrogel particle was ground to an average diameter of 50 μm, and 10 mg of powder was suspended in 1 mL of distilled water. Surface electric charge was determined by measuring the zeta potential with a zeta potential analyzer (Zetasizer Nano-ZS; Malvern Instruments, Southborough, UK).

All experiments were repeated three times. Data between the five groups were compared by analysis of variance (ANOVA) and Tukey–Kramer test with a significance level of $p < 0.05$.

1.1.2 Preparation of poly-HEMA/TMPT hydrogels containing CPC by immersion-loading methods

CPC powder (Wako, Osaka, Japan) was dissolved in distilled water to prepare 500 μg/mL of solution. Thirty milligrams of each of the five hydrogel particles was immersed in 30 μL of CPC solution (total amount of CPC, 15 μg) in a glass tube, and stored at 25°C for 24 h to take up CPC.

To determine the amounts of CPC adsorbed by the prepared particles, the residual solution in the glass tube after loading was diluted with water and the amounts of residual CPC (A_r) were calculated from the concentration of CPC measured by

high-performance liquid chromatography (HPLC). The HPLC system (Prominence; Shimadzu, Kyoto, Japan) used a reverse-phase column (Puresil 5μ C18 column; Waters, Millford, MA, USA). Acetonitrile and 5 mM phosphate buffer solution including 100 mM sodium perchlorate mixed at 70/30 (vol/vol) were used for the mobile phase at a flow rate of 1 mL/min, and readings were performed at 260 nm. The lowest limit of detection for CPC was 1.0 μg/mL. The amount of CPC adsorbed (A_p) in micrograms was calculated by subtracting A_r from 15.

To determine the amounts of CPC strongly bound to the hydrogel (As), the CPC-loaded hydrogel was washed in 500 μL of water to remove loosely bound CPC. The amounts of CPC liberated into the water (A_w) were then measured and the value of A_s was calculated as; A_s (μg) = 15 − A_r − $A_{w.}$ The experiments were repeated five times.

1.1.3 Determination of release of CPC

Thirty milligrams of non-washed or washed hydrogel particles loaded with CPC were placed in one well of a 48-well microplate, and 500 μL of distilled water was added. The eluent was replaced after storage at 37˚C for 12, 24, 48, and 72 h, and the concentration of CPC released determined by HPLC. The experiments were repeated five times. Data between the two groups were statistically analyzed by the Student's *t*-test with a significance level of $p < 0.05$, and the results of the five groups were compared by ANOVA and Tukey–Kramer test with a significance level of $p < 0.05$.

1.1.4 Analysis of the mechanism of CPC binding to poly-HEMA/TMPT hydrogels

To analyze the mechanism of CPC binding to hydrogels, release of CPC from washed

particles into three different elution media was determined. Non-ionic detergent, an electrolyte NaCl solution, and urea solution were used to examine the relevance of hydrophobic interaction, electrostatic interaction, and hydrogen bonding, respectively. Thirty milligrams of washed hydrogel particles were immersed in 500 μL of 0.1 M Triton X-100 (Nacalai Tesque, Kyoto, Japan), 0.1 M NaCl solution (Wako), or 0.1 M urea solution (Wako), and stored at 37°C. Replacing the elution medium after 12, 24, 48, 72, 96, and 120 h, the concentration of CPC eluted into each solution was measured by HPLC and the total amounts of CPC released were calculated. The experiments were repeated five times, and the results were statistically analyzed by the Student's *t*-test with a significance level of $p < 0.05$.

1.2 Results

1.2.1 Characterization of poly-HEMA/TMPT hydrogels

Figure 2 shows a microscopic image of H50 particles. All of the hydrogel particles fabricated were transparent and had irregular shape. Water absorbability, hydrophobicity, and surface electric charge of each hydrogel particle are shown in Fig. 3. The H70 and H90 particles with higher HEMA contents showed significantly greater water absorption than H10. The hydrophobicity represented by the *H*-index increased with HEMA content, and a significant increase was observed amongst H30–H90 ($p < 0.05$, ANOVA, Tukey–Kramer test). All of the hydrogels had negative charges, with zeta potential values of −25 to −31 mV. The negative values for H50, H70, and H90 were significantly less than those of H10 and H30 ($p < 0.05$, ANOVA, Tukey–Kramer

test).

1.2.2 Amounts of CPC adsorbed

The amounts of CPC adsorbed to each hydrogel particle as prepared (Ap) and after washing (As) are summarized in Table 2. Adsorption ratios (%) for the particle as prepared (R_p) and after washing (R_s) were also calculated. All five particles showed high adsorption ratios when measured as prepared, and complete adsorption of CPC in immersed solution was observed for the particles with HEMA at 50% or more.

After washing, the adsorption ratios for H10 and H30 were greatly reduced, indicating that most of CPC was loosely bound to the surfaces of these particles. In contrast, H50, H70, and H90 demonstrated high adsorption ratios for CPC even after washing, and adsorption of more than 97% of CPC was observed for H70 and H90.

1.2.3 Release of CPC from hydrogels

Concentrations of CPC released from immersion-loaded hydrogel particles into water are shown in Fig. 4. For the particles as prepared, at the initial stage after 12 h, H10 and H30 showed significantly greater release than other particles ($p < 0.05$, ANOVA, Tukey–Kramer test). However, the release concentrations from H10 or H30 were reduced to around 2 μg/mL after 24 h and were nil (below the level of detection limit of 1.0 μg/mL) after 48 or 72 h, respectively. H70 and H90 showed release of CPC at less than 0.8 μg/mL after 12 h and no release was detected thereafter. H50 released around 4.5 μg/mL of CPC after 12 h. Amongst the five hydrogels, only H30 and H50 showed release of CPC until 48 h.

The CPC-release after 12 h from washed H10 and H30, which showed low

adsorption ratios, was significantly lower than those from the particles as prepared ($p < 0.05$, ANOVA, Tukey–Kramer test). Washed H70 and H90 demonstrated small amounts of CPC release, similar to the particle without washing. Initial CPC release from H50 after washing was not significantly different from those of the specimen as prepared ($p > 0.05$, Student's *t*-test), and washed H50 demonstrated the release of CPC until 48 h.

Table 3 shows the total amounts of CPC released into water. Release rates as a percentage of the amounts adsorbed into each hydrogel was also calculated. For both of the particles as prepared and washed after loading, a large part of the CPC adsorbed was found to be released from H10 and H30. In contrast, for H70 and H90, only a small amount of CPC was released and most of loaded CPC remained trapped within the particles. For H50, about 30% of loaded CPC was released, regardless of washing procedure.

1.2.4 Analysis of mechanism of CPC binding to poly-HEMA/TMPT hydrogels

Total amounts of CPC released from each hydrogel particle into water (control), Triton X-100, NaCl, or urea aqueous solutions are shown in Fig. 5. The specimens H30–H90 demonstrated significantly greater release of CPC into Triton X-100 compared with water, and remarkable increases were observed for H50, H70, and H90 ($p < 0.05$, Student's *t*-test). Based on the amounts of adsorbed CPC determined (Table 2), it was found that almost all of CPC loaded was released into Triton X-100 from all hydrogels. On the contrary, the amounts of CPC released into NaCl solution were significantly decreased compared with that released into water for all hydrogels ($p < 0.05$, Student's *t*-test). When the release concentrations into urea solution were compared with those released into water, no significant difference was observed for H10 and H30, but H50,

H70 and H90 exhibited significantly lower release ($p < 0.05$, Student's *t*-test).

1.3 Discussion

Several kinds of hydrogels for drug release have been reported, such as gelatin, polysaccharide, polyHEMA, or poly(ethylene glycol) gels [27-33]. Because many reconstructive materials for dental use are based on methacrylate resins, a non-biodegradable polyHEMA gel is expected to be compatible with dental resins. Polymers based on the hydrophilic monomer HEMA [34-36] can take up water-soluble drugs [24] owing to its water absorbability [23]. However, most of the HEMA-based hydrogels previously reported are homopolymers of HEMA or copolymers of HEMA and a mono-metharylate such as methyl methacrylate [37-39]. Those hydrogels consisting of single-chain polymers are too elastic to be pulverized and have limited formability. Therefore, in this study, hydrogels were fabricated by combining HEMA and a cross-linking monomer TMPT. TMPT is a tri-functional methacrylate that has been used for dental resin composites [40-43]. The cross-linking ability of TMPT is considered to be effective for increasing the mechanical strength of a hydrogel, providing a greater possibility for application to various reconstructive materials.

For the binary system of HEMA and TMPT, an increase in HEMA content resulted in greater water absorbability. This means that the hydrophilic hydroxyl group of polymerized HEMA plays a role in absorbing water. It is known that the total water absorbed by a hydrogel comprises bound and free water [23]. When a dry hydrogel begins to absorb water, the first water molecules entering the polymer matrix hydrate the most hydrophilic groups. As the hydrophilic groups are hydrated, the network swells

and hydrophobic groups are exposed. The network absorbs additional water, but this additional swelling is opposed by cross-links, and the hydrogel reaches an equilibrium swelling level. The additional swelling water that is absorbed beyond the total bound water is called free water, and is assumed to fill the space between the network chains. The results of water absorption tests (Fig. 3A) reflect the swelling of the polymer network, suggesting less cross-linking levels for hydrogels with smaller TMPT content. Accordingly, hydrogels with greater HEMA content can contain more bound and free water, resulting in greater water absorbability.

Conversely, from the results of *H*-index measurements, the hydrogels with higher ratios of HEMA showed greater hydrophobicity (Fig. 3B). It has been reported that the *H*-index, which has a similar conceptual basis to the octanol-water partition coefficient (logP) used to classify molecular hydrophobicity, is useful to detect differences in the hydrophobicity of hydrogels [44, 45]. Alkyl chains of the polymerized HEMA in the network possibly act to provide hydrogels with a hydrophobic nature. Binding of the hydroxyl group of HEMA with some other part of the polymer may also decrease the hydrophilic nature of the hydrogels.

To evaluate the surface electric charge of each poly-HEMA/TMPT hydrogel, the zeta potential was measured. All five hydrogels fabricated had a negative charge. Jang *et al.* [46] and Laverty *et al.* [47] reported that HEMA possesses an anionic character, which is present in its polymeric form via the polar hydroxyl groups. Our results were in accordance with these previous findings. However, the negative values for H50, H70, and H90, with greater HEMA contents, were significantly lower than those of the H10 and H30. Accordingly, the surface electric charge of the hydrogels prepared was not dependent upon the ratio of HEMA.

The quaternary ammonium compound CPC was chosen as the antimicrobial to be loaded into the hydrogels. CPC is highly soluble in water (50 mg/mL at 20 °C) and has a strong antimicrobial activity against oral bacteria and fungi [48-52]. In general, the amounts of water-soluble drug loaded into the cross-linked hydrogel increase, as cross-linking density decreases and as the water absorption of the hydrogel increases [53]. Our results confirmed that the hydrogel particles with HEMA at 50% or more demonstrated greater adsorption of CPC than H10 and H30. In addition, the adsorption ratio for H10 and H30 was greatly reduced by washing, indicating that most of CPC was loosely bound to these particles. In contrast, H70, and H90 demonstrated high adsorption, of more than 97% of the initial CPC even after washing, indicating that CPC was strongly adsorbed to these hydrogels.

Water-soluble drugs are released from the hydrogel by passive diffusion, which is the most common release mechanism [54]. From the results for the total amount of CPC released into water and the release rates (Table 3), for both particles as prepared and those washed after loading, a large part of the adsorbed CPC was found to be released from H10 and H30 but only a small amount was released from H70 and H90. Amongst the five hydrogels, H50 after washing showed the largest amounts of release, for the longest period. It is interesting to note that release characteristics of CPC into water did not correlate with the water absorbability of the hydrogels.

To analyze the binding mechanism of CPC to as-prepared hydrogels, Triton X-100, NaCl solution, or urea solution were used as elution media and the release of CPC was evaluated. Hydrogels H30–H90 demonstrated significantly greater release of CPC into the non-ionic surfactant solution Triton X-100 than into water. Because the surfactant hinders hydrophobic interaction [55-57], CPC adsorbed to hydrogels by hydrophobic

interaction is desorbed by Triton X-100 and released. Amongst the five hydrogels, remarkable increases in release into Triton X-100 were observed for H50, H70, and H90, which have high hydrophobicity. These results indicate that CPC is bound to the polymer network through hydrophobic interactions. CPC is a cationic antimicrobial agent, with a positive charge on the N atom of the pyridinium ring. It is well known that this compound shows strong interaction with negatively charged substances [47]. However, release of CPC from the hydrogels into NaCl solution was not increased compared with that into water. Moreover, H50, H70, or H90, which have lower negative charges, demonstrated release in smaller amounts into NaCl solution. Accordingly, it is unlikely that electrostatic interaction is involved in the binding of CPC to the HEMA/TMPT polymer network. Zahedi *et al.* [58] reported involvement of hydrogen bonding between water-soluble drugs and the hydroxyl groups of polyHEMA hydrogels in drug-loading. However, in the present study, the amounts of CPC released from hydrogels into urea solutions did not significantly differ from those into water, suggesting that hydrogen bonding has no relevance in the binding of CPC to poly-HEMA/TMPT. Thus, it is considered that CPC taken up by hydrogels binds to the polymer frame mainly through hydrophobic interactions.

The loading of CPC and its release from fabricated poly-HEMA/TMPT hydrogels can be explained by the state of CPC, polymer mesh sizes, and hydrophobicity of the polymer (Fig. 6). A cationic surfactant CPC forms micelles by hydrophobic interactions at concentrations above 322 μg/mL (0.9 mM) [59-61]. In the present study, CPC in the small micellar state was taken up by the hydrogels as they were immersed into 500 μg/mL of CPC solution, which is slightly greater than the critical micelle concentration. The average size of such CPC micelles is about 1 nm [62]. Conversely, hydrogels with

greater amounts of HEMA have a larger polymer network mesh size [63, 64]. Small amounts of CPC micelles are adsorbed to the superficial part of the polymer in H10, which has the smallest mesh size amongst the five hydrogels, and most of the adsorbed CPC is rapidly released within a short period. For H30, which has a larger polymer mesh size than H10, more micellar CPC is adsorbed to the polymer, and a longer release of CPC is possible than from H10. As for H70 and H90, which have higher water absorbability and larger mesh sizes, a greater amount of CPC than those for H10, H30, and H50 is trapped more deeply within the polymer. However, CPC taken up by these polymers is desorbed less because of strong adsorption by hydrophobic interaction. Amongst the five hydrogels, H50 has an optimal mesh size for penetration of micellar CPC and a binding affinity with CPC, showing the largest amounts of release, for the longest period. It has been reported that the critical micelle concentration of CPC was decreased in NaCl aqueous solution [65]. Therefore, in the presence of NaCl, CPC micelle formation was promoted in the hydrogels and resulted in production of larger micelles. This may be the reason for decreased amounts of CPC released from the hydrogels into NaCl solutions than into water.

1.4 Conclusions

New, non-biodegradable poly-HEMA/TMPT hydrogels, loaded with CPC by immersion into CPC solutions, demonstrated release of the agent in water. Amongst hydrogels with different ratios of HEMA and TMPT, a 50% HEMA/50% TMPT specimen was found to be the most suitable for loading and release of CPC.

CHAPTER 2

Evaluation of the ability of poly-HEMA/TMPT hydrogels to recharge CPC

2.1. Materials and methods

2.1.1 Measurement of minimum inhibitory concentration values of CPC against oral bacteria and fungi

Minimum inhibitory concentrations (MIC) against four oral bacterial species and one fungus were determined by a microdilution assay. For the measurement, *Streptococcus mutans* UA159 and NCTC10449, *Lactobacillus casei* ATCC4646, *Enterococcus faecalis* SS497, and *Candida albicans* SC5314 were used.

Serial 2-fold dilutions of 50 μL of CPC solution were prepared in the wells of a 96-well microplate, and 50 μL of microbial suspension at approximately 2×10^6 colony-forming units (CFU)/mL was inoculated into each well. The plates were incubated anaerobically for 48 h at 37°C, and MIC values were determined as the lowest concentration in the well at which no turbidity was observed by visual examination. The tests were repeated five times for each strain.

2.1.2 Assessment of ability of poly-HEMA/TMPT hydrogels to recharge CPC

Thirty mg of H30 or H50 particles loaded with CPC were immersed in 500 μL of distilled water for 48 h to make CPC release, and then 30 μL of CPC solution at 500 μg/mL was added to the particles. After storage at 37°C for 24 h, the release of CPC was re-evaluated, as described in Chapter 1. The procedure for recharging and the measurement of CPC-release were run in three consecutive series, and the experiments

were repeated five times.

The profiles of CPC-release from the hydrogel particles under different recharging protocols were also analyzed. For this test, H50 particles, which showed release of CPC for the longest period amongst the five hydrogels in previous experiments in Chapter 1, were used. The CPC-loaded H50 was prepared by immersion of 30 mg of H50 particles into 30 μL of CPC solution at 500 μg/mL or 5 mg/mL. The release of CPC was carried out once by immersion of each hydrogel into distilled water for 48 h, and recharging was conducted using the CPC solutions at the same concentrations as those used for loading. The durations of immersion to recharge with CPC were 5 min, 6 h, or 12 h. The release of CPC from recharged H50 was evaluated in three repeated procedures as described above. The experiments were repeated five times.

2.1.3 CPC-loading to hydrogels by pre-mixing methods and analysis of release characteristics

The loading of CPC was performed by mixing CPC powder into the HEMA/TMPT monomer before polymerization and the release profile was evaluated. To a monomer mixture of H50 (HEMA/TMPT = 50/50 wt%), CPC powder was added at 0.5, 5, or 10 wt%, and the monomer was heat polymerized. The prepared polymer was ground to obtain an average diameter of 500 μm. Thirty milligrams of CPC-pre-mixed hydrogels were placed in one well of a 48-well microplate, and the concentration of CPC released into 500 μL of distilled water was measured by HPLC, after replacing eluent at certain periods. The experiments were repeated five times.

To compare the release characteristics based on two different loading methods; i.e.,

immersion loading and pre-mix loading, release of CPC into water from H50 loaded with CPC by immersion in a 0.5% (5 mg/mL) solution was also determined.

2.1.4 Release of CPC from pre-mixed hydrogels after recharging

After immersion of 30 mg of 0.5% CPC-pre-mixed H50 particles in distilled water for 72 h to make CPC release, recharging of CPC was conducted by immersion in 30 µL of CPC solution at 500 µg/mL for 5 min. The release of CPC was then evaluated again, as described in Chapter 1. The procedure for recharging and the measurement of CPC release were run in three consecutive series, and the experiments were repeated five times. The results were statistically analyzed by the Student's *t*-test with a significance level of $p < 0.05$.

2.2 Results

2.2.1 MIC values of CPC

For all microorganisms, the same endpoint was obtained for each of the five replicates. The MIC value of CPC against *S. mutans* NCTC10449 was 0.8 µg/mL. Against *S. mutans* UA159, *L. casei* ATCC4646, *E. faecalis* SS497, and *C. albicans* SC5314, the value was determined to be 1.6 µg/mL.

2.2.2 Ability of poly-HEMA/TMPT hydrogels to recharge CPC

For both H30 and H50, the release concentrations were recovered by the recharging process (Fig. 7). The release profile after recharging was similar to that after the first

loading, showing reduced release amounts at 48 h. Repeated recharging every 48 h resulted in maintenance of CPC levels above 2 μg/mL for H50 particles.

Release profiles under different recharging protocols are shown in Fig. 7. For a 5-min recharge, although the use of 500 μg/mL CPC solution was not effective to recover the release concentrations, continuous release of CPC above 1.6 μg/mL was obtained by recharge every 3 days with a 5 mg/mL CPC solution (Fig. 8A). In the case of recharging for longer times of 6 and 12 h (Fig. 8B, 8C), over 1.6 μg/mL of CPC release was maintained by recharging every 2 days with CPC solution at 500 μg/mL and every 3 days with 5 mg/mL solution.

2.2.3 Release characteristics of CPC-loaded hydrogels prepared by pre-mixing methods

A H50 hydrogel loaded with CPC by immersion into 0.5% CPC solution demonstrated 21.8 μg/mL of CPC release after 12 h, but the release continued only for 5 days (Fig. 9A). On the contrary, while the initial release concentration was significantly lower than that of the immersion-loaded hydrogel, a H50 hydrogel pre-mixed with 0.5% CPC powder demonstrated release of CPC until 20 days, and release over 1.6 μg/mL was maintained until 10 days (Fig. 9A).

Release of CPC at high concentrations after 12 h, i.e., 175 and 860 μg/mL, was observed for 5 and 10% pre-mixed specimens, respectively (Fig. 9B). Although the CPC release was reduced gradually, both materials exhibited sustained release of CPC over 120 days.

To compare the release kinetics from 0.5% CPC-pre-mixed H50 with that of H50 loaded with CPC by immersion into a 5 mg/mL (0.5%) solution, modeling of drug

release profiles was performed using a simple power law-based equation, derived by Ritger and Peppas [66, 67].

$$M_t / M_\infty = Kt^n,$$

where M_t and M_∞ are the absolute cumulative amounts of drug released at time t and infinite time, respectively. M_t/M_∞ is typically applied to the first 60% of total drug released. K is a constant that incorporates both the structural and geometric characteristics of the device, whereas the release exponent (n) indicates the mechanism of drug release. The above equation can be modified to a logarithmic function, and when the $\log_{10}$ fraction of total drug released is plotted against the $\log_{10}$ time, the release exponent (n) is equivalent to the gradient of these line plots.

$$\log_{10}(\%\ \text{released}) = \log_{10}(M_t / M_\infty) = \log K + n \log_{10} t$$

Figure 10 shows the $\log_{10}$ fraction of total drug released from a 0.5% CPC-immersion-loaded or pre-mixed hydrogel against $\log_{10}$ time. A value of n less than 0.43 indicates the occurrence of Fickian diffusion. A value of n between 0.43 and 0.85 indicates non-Fickian transport. When the n value is greater than 0.85, there is zero-order release, which means release occurs at a constant rate. The value of n for a CPC-loaded hydrogel by the immersion method was 0.21 so was found to demonstrate Fickian diffusion pattern, while the 0.5% CPC-pre-mixed hydrogel, with n = 0.668, exhibited non-Fickian transport.

2.2.4 Release of CPC from pre-mixed hydrogels after recharging

An increase in the concentrations of CPC release was observed after re-immersion of CPC-pre-mixed H50 into 500 µg/mL CPC solution for 5 min (Fig. 11). The release profile for a 0.5% CPC-immersion-loaded hydrogel with 5-min recharging was

extrapolated into Fig. 11. The CPC release from an immersion-loaded hydrogel gradually decreased. On the contrary, concentrations of CPC released from pre-mixed hydrogels were maintained at over 2 μg/mL by recharging, showing significantly greater values than immersion-loaded hydrogels at all time periods after 3 days ($p < 0.05$, Student's *t*-test).

2.3 Discussion

To design the CPC recharging protocol, we determined the minimum inhibitory concentrations (MIC) values of CPC against several oral bacteria and a fungus, such as *S. mutans*, *L. casei*, *E. faecalis*, and *C. albicans*. *S. mutans* and *L. casei* are caries-related bacteria [68, 69], and *E. faecalis* is frequently isolated from infected root canal [70-73]. *C. albicans* is strongly related to denture-induced stomatitis [74-77]. The MIC values of CPC determined for these microorganisms were 1.6 μg/mL or less. Previous studies described similar MIC values (less than 1.6 μg/mL) for CPC against *S. mutans* MT8148, *S. salivarius*, and *S. mitis* [12, 78, 79]. Based on this information, the release concentration of CPC at 1.6 μg/mL or over was set as a value for subsequent study to evaluate the effectiveness of recharging to hydrogels.

For both H30 and H50, it was found that CPC-release concentrations could be recovered by exposure to CPC solution for 24 h. The release profile after every recharging process was similar to that after the first loading. In addition, H50 demonstrated continuous release above the effective concentration of CPC through recharging. These results indicate that repeated loading of CPC to a polymer consisting of 50% HEMA/50% TMPT was possible. Andrade-Vivero *et al.* [25] and Laverty *et al.*

[47] reported that release of drug for longer periods from polyHEMA gels was obtained when they were immersed in higher concentrations of drug solution. Using H50, we further analyzed the release profiles of CPC under several recharging protocols with combinations of different CPC concentrations and exposure periods. The results suggest that CPC solutions at higher concentrations, such as 5 mg/mL (0.5%), are needed in the case that a short period exposure of 5 min is adopted. It was also confirmed that sustained release of CPC above 1.6 μg/mL can be achieved by recharging with a 500 μg/mL (0.05%) solution over a longer period, such as 6 h. Ehara *et al.* [80] reported that a methacrylic acid (MAA)-based resin to which CPC was bound and could be recharged through an ion-exchange mechanism after release. They demonstrated that recharge of CPC into this resin was possible by immersion into 2.5 mg/mL of CPC solutions for 30 min. Cao *et al.* [81] also reported re-adsorption of chlorhexidine to a co-polymer of MAA with diurethane dimethacrylate by exposure of the specimen to a 10% chlorhexidine solution for 24 h. Our system of recharging the poly-HEMA/TMPT hydrogels by diffusion of CPC is considered to be advantageous, because sustained release of CPC can be achieved by a shorter recharge period of 5 min, using a 5 mg/mL (0.5%) CPC solution.

When CPC was repeatedly taken up by poly-HEMA/TMPT hydrogels by immersion into CPC solutions, concentrations of CPC released were decreased in 48 h and was zero after 72 h, even for the H50 hydrogel. Therefore, CPC powder was mixed with the monomer components of H50, and the release profile of CPC from the polymerized hydrogel was evaluated. A hydrogel pre-mixed with 0.5% CPC demonstrated release of CPC until 20 days, and release above 2 μg/mL was maintained for 10 days. With 5 and 10% CPC-pre-mixed hydrogels, release of CPC continued for

over 120 days. It has been reported that methacrylate resins incorporated with chlorhexidine powder before polymerization exhibited release of the agent for 4–5 weeks [82, 83]. Thus, pre-mixing of the drug with monomers is an effective method to attain long-period release from the polymer. Analysis using the equation derived by Ritger and Peppas revealed that CPC-immersion-loaded hydrogels showed a release pattern corresponding to Fickian diffusion with an initial burst. In contrast, the release profile from a CPC-pre-mixed hydrogel showed non-Fickian diffusion. Non-Fickian transport means that there is an anomalous release pattern, with complex drug release kinetics under control of both drug diffusion and polymer relaxation [38, 47]. Through the pre-mixing method, CPC can be dispersed homogeneously inside the polymer and such a structure for CPC-carriage may be one of the reasons for the specific release pattern. In addition, it is considered that CPC dissolved in a monomer mixture of HEMA and TMPT does not form micelles as it does in water. It is possible that binding of non-micellar CPC to the HEMA/TMPT polymer by hydrophobic interaction is stronger and therefore long-term release with no initial burst is attained.

Immersion of a CPC-pre-mixed H50 into a CPC solution was effective to recover CPC release, and recharge of CPC was possible, similar to the immersion-loaded hydrogels. Furthermore, for the 0.5%-CPC-pre-mixed hydrogel, release of CPC above 2 μg/mL was maintained by short period recharges of 5 min using a CPC solution at 500 μg/mL. The release of CPC freshly adsorbed by recharging, in addition to the initially loaded CPC, resulted in constant release at a level that was effective against oral bacteria and fungi. A combination of pre-mix loading of CPC powder and recharge with CPC solution, therefore, is the most appropriate and efficient way to obtain sustained release of CPC.

From the release profile of pre-mixed H50 after recharging, it is likely that pre-mixing of CPC powder did not affect basic characteristics of H50 polymer including hydrophilicity, hydrophobicity, and mesh size. However, in general, addition of non-polymerizable components into resins compromises curing behavior [82, 84]. Therefore, to apply the poly-HEMA/TMPT hydrogels to various methacrylate resins, further study to compare the mechanical properties of H50 and CPC-pre-mixed H50 is needed.

In the oral environment, proteins in the saliva adsorb and cover the surface of reconstructive materials [85-88]. To clarify the usefulness of the hydrogels for application to dental resins, their ability to release and recharge CPC in the presence of saliva remains to be determined.

Russell [89] reported that frequent use of water-soluble antimicrobials resulted in development of tolerance in *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas stutzeri*. So far, no study is available which investigated acquirement of resistance to antimicrobials by oral bacteria. Preliminary examination conducted by our group demonstrated that exposure of *S. mutans* or *E. faecalis* to CPC at sub-MIC concentrations did not cause any resistance (data not shown). Usage of CPC appears to be effective in terms of longevity of antimicrobial effects, but such advantage needs to be clarified in detail.

Another important issue raised for continuous release of antimicrobials is possible negative influence on oral microbial homeostasis. The presence of a resident microflora prevents disease by reducing the chance of colonization by exogenous species. The use of antimicrobials may perturb the resident microflora resulting in overgrowth by drug-resistant, but previously minor, components of the oral microflora, or colonization

by exogenous and potentially pathogenic organisms [90]. For examining clinical benefits and risk, *in vivo* examination of sustained CPC-release systems developed must be conducted.

2.4 Conclusions

Recharge of CPC into the 50% HEMA/50% TMPT hydrogel was possible and continuous release of CPC above the effective antimicrobial concentration was attained. CPC-loaded hydrogels prepared by a pre-mixing method exhibited an extended period of initial CPC release, and a sustained release system for CPC may be achieved by combining an appropriate recharge protocol with pre-mixed hydrogels.

GENERAL CONCLUSIONS

Reconstructive materials with sustained antimicrobial effects could be useful for preventing infectious diseases in an environment containing indigenous bacteria and fungi such as the oral cavity. With the objective of applying a non-biodegradable hydrogel to resin-based materials as a reservoir for water-soluble antimicrobials, novel hydrogels consisting of HEMA and TMPT were fabricated, and CPC was loaded into the poly-HEMA/TMPT hydrogels.

This *in vitro* study confirmed that a poly-HEMA/TMPT hydrogel comprising 50% HEMA/50% TMPT could be effectively loaded and recharged with CPC by immersion into a CPC solution, demonstrating the longest release of CPC, above the concentration required to inhibit oral bacteria and fungi. The binding of CPC to the hydrogels was mainly through hydrophobic interaction among five hydrogels with different ratio of HEMA/TMPT. Additionally, loading of CPC into a hydrogel by mixing CPC powder with the HEMA/TMPT monomer before polymerization resulted in marked extension of the initial CPC-release period. It is possible to achieve a sustained release system for antimicrobials by pre-mix loading and recharging CPC into a 50% HEMA/50% TMPT hydrogel.

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TABLE LEGENDS

Table 1. Hydrogels fabricated and their monomer compositions.

Table 2. Amounts of CPC adsorbed to hydrogel particles and adsorption ratio.

Table 3. Total amount of CPC released and release rates.

FIGURE LEGENDS

Fig. 1. Monomers used.
A: 2-hydroxyethyl methacrylate (HEMA)
B: Trimethylolpropane trimethacrylate (TMPT)

Fig. 2. Microscope image of H50 hydrogel particles.

Fig. 3. Water absorbability (A), hydrophobicity (B), and surface electric charge (C) of poly-HEMA/TMPT hydrogels.
The bar represents the standard deviation of three replicates. a, b, c, d: No significant differences between the same letters (ANOVA, Tukey–Kramer test, $p < 0.05$).

Fig. 4. Concentrations of CPC released into water from each hydrogel.
A: particles as prepared, B: washed particles. The bar represents the standard deviation of five replicates.

Fig. 5. Total amount of CPC released from hydrogel particles into water (control), Triton X-100, NaCl, or urea solutions.
The bar represents the standard deviation of five replicates.

Fig. 6. Schematic diagram for loading of CPC and its release from five poly-HEMA/TMPT hydrogels prepared.

Fig. 7. CPC-release profile of H30 and H50 with recharging.
The bar represents the standard deviation of five replicates.

Fig. 8. CPC-release profiles for H50 under different recharging protocols.
Recharging time A: 5 min, B: 6 h, C: 12 h. The bar represents the standard deviation of five replicates.

Fig. 9. CPC-release from hydrogels prepared by pre-mixing method.
A: 0.5% CPC-immersion-loaded and CPC-pre-mixed hydrogel, B: 5% and 10% CPC-pre-mixed hydrogels. The bar represents the standard deviation of five replicates.

Fig. 10. The $\log_{10}$ fraction of total drug released from 0.5% CPC-immersion-loaded or pre-mixed hydrogels against $\log_{10}$ time.

Fig. 11. CPC-release profile of 0.5% CPC-immersion-loaded and CPC-pre-mixed hydrogels with recharging.
The bar represents the standard deviation of five replicates.

Table 1. Hydrogels fabricated and their monomer compositions.

Abbreviation	HEMA / TMPT (wt%)	HEMA / TMPT (mol%)
H10	10 / 90	23 / 77
H30	30 / 70	53 / 47
H50	50 / 50	72 / 28
H70	70 / 30	86 / 14
H90	90 / 10	96 / 4

Table 2. Amounts of CPC adsorbed to hydrogel particles and adsorption ratio.

	Amount of CPC adsorbed as prepared (A_p)	Adsorption ratio (R_p) calculated from A_p	Amount of CPC adsorbed after washing (A_s)	Adsorption ratio (R_s) calculated from A_s
H10	13.89 (0.20)	92.58 (1.33)	2.06 (0.51)	13.74 (3.36)
H30	14.28 (0.40)	95.23 (2.68)	2.59 (0.72)	17.27 (4.81)
H50	15.00	100	11.74 (0.28)	78.24 (1.89)
H70	15.00	100	14.62 (0.34)	97.49 (2.29)
H90	15.00	100	14.87 (0.29)	99.13 (1.95)

μg, ( ) : S.D., n = 5.

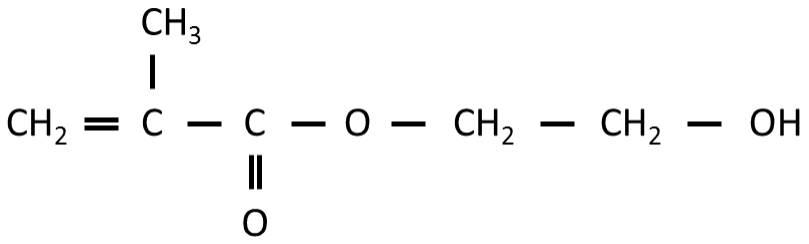
R_p (%) = A_p / 15 ×100, R_s (%) = A_s / 15 × 100.

Table 3. Total amount of CPC released and release rates.

	Particles as prepared		Washed particles	
	Amount of CPC released (μg)	Release rates (%)	Amount of CPC released (μg)	Release rates (%)
H10	10.25 (0.64)	73.82	1.97 (0.55)	95.71
H30	9.91 (0.83)	69.41	2.36 (0.50)	91.30
H50	3.51 (0.58)	23.38	3.34 (0.75)	28.47
H70	0.39 (0.35)	2.59	0.58 (0.33)	3.96
H90	0.14 (0.31)	0.91	0.43 (0.39)	2.89

( ) : S.D., n = 5.

A



B

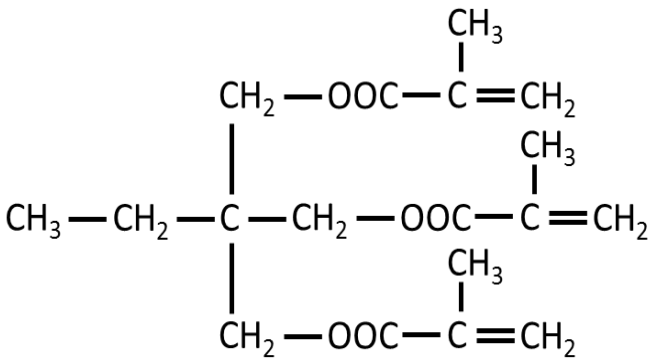


Fig. 1. Monomers used.
A: 2-hydroxyethyl methacrylate (HEMA)
B: Trimethylolpropane trimethacrylate (TMPT)

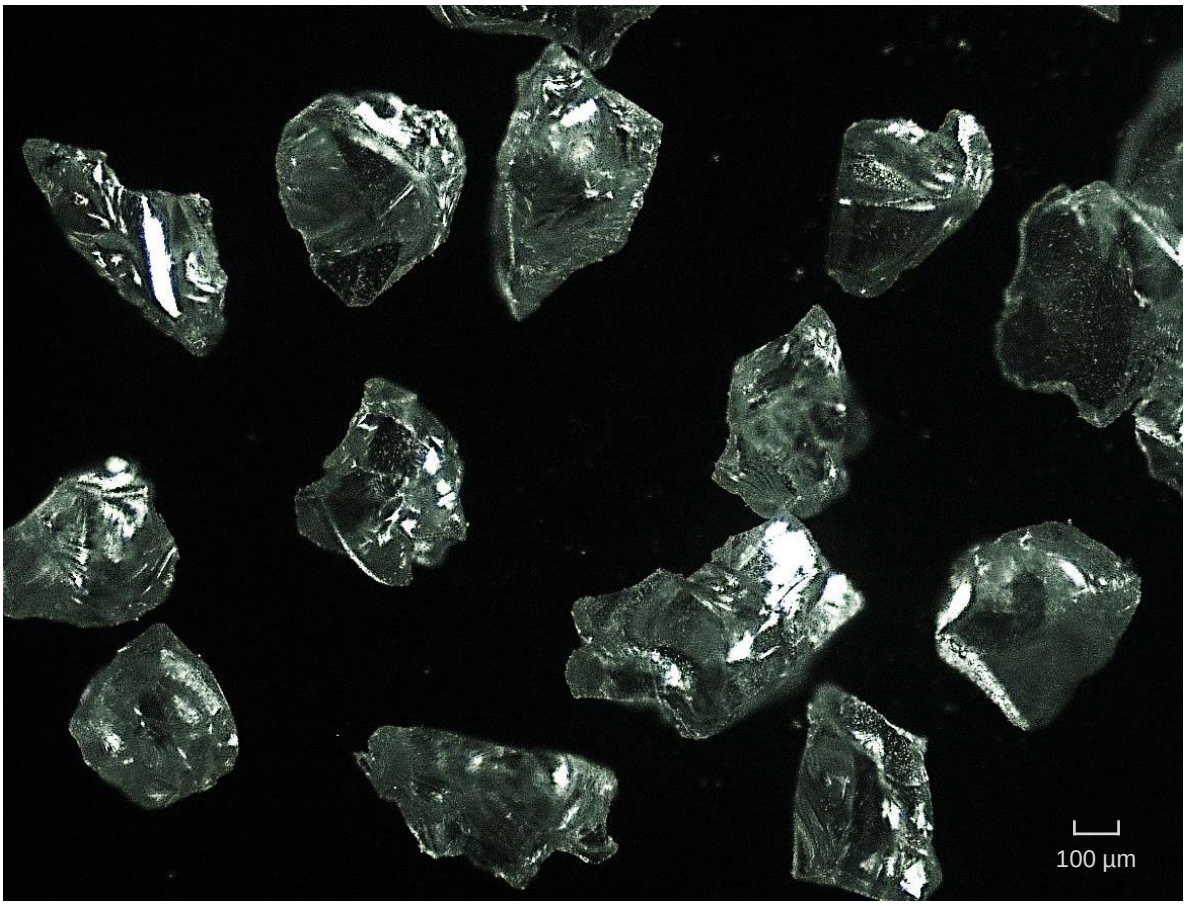


Fig. 2. Microscope image of H50 hydrogel particles.

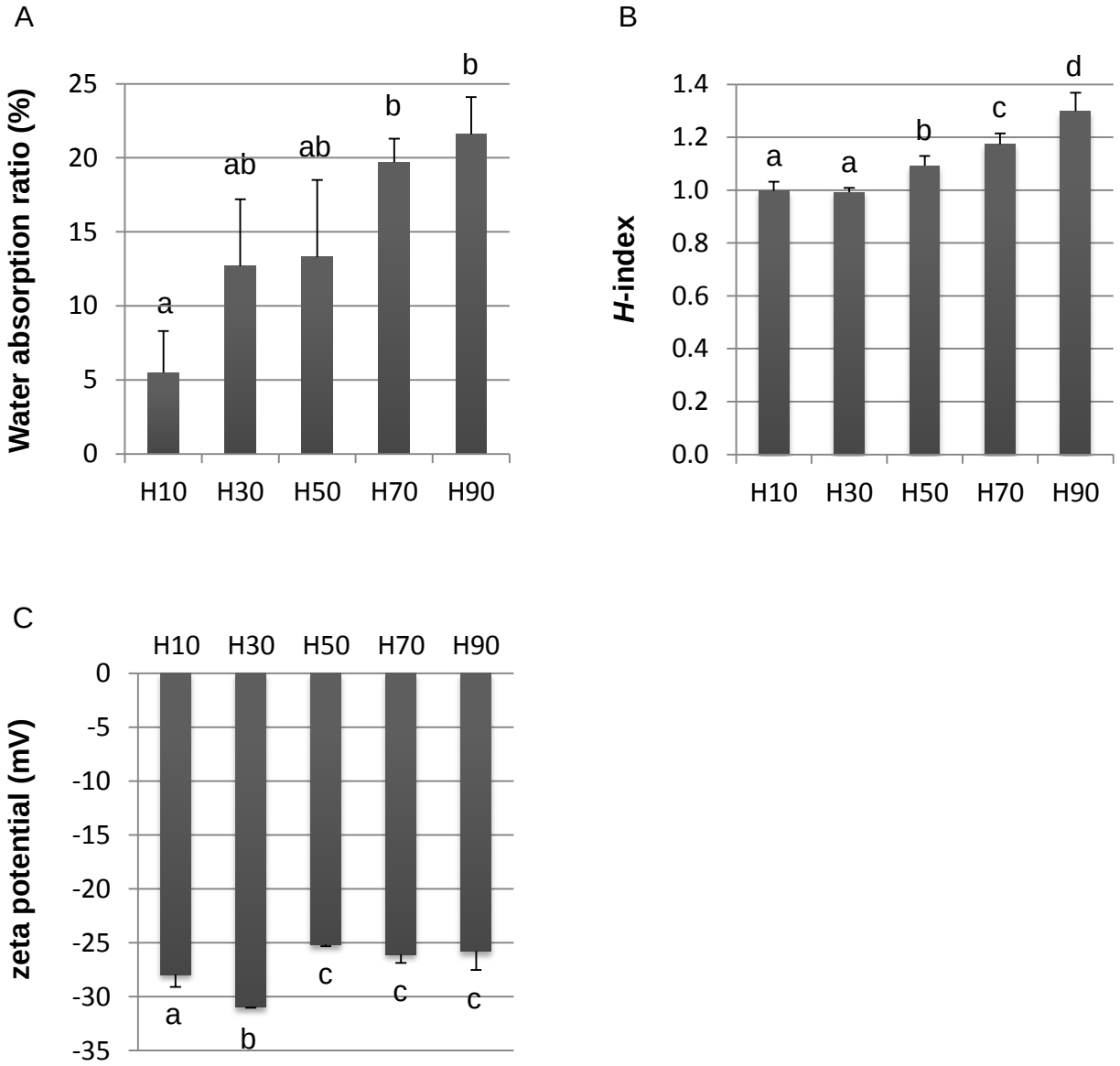


Fig. 3. Water absorbability (A), hydrophobicity (B), and surface electric charge (C) of poly HEMA/TMPT hydrogels.

The bar represents the standard deviation of three replicates. a, b, c, d: No significant differences between the same letters (ANOVA, Tukey–Kramer test, $p < 0.05$).

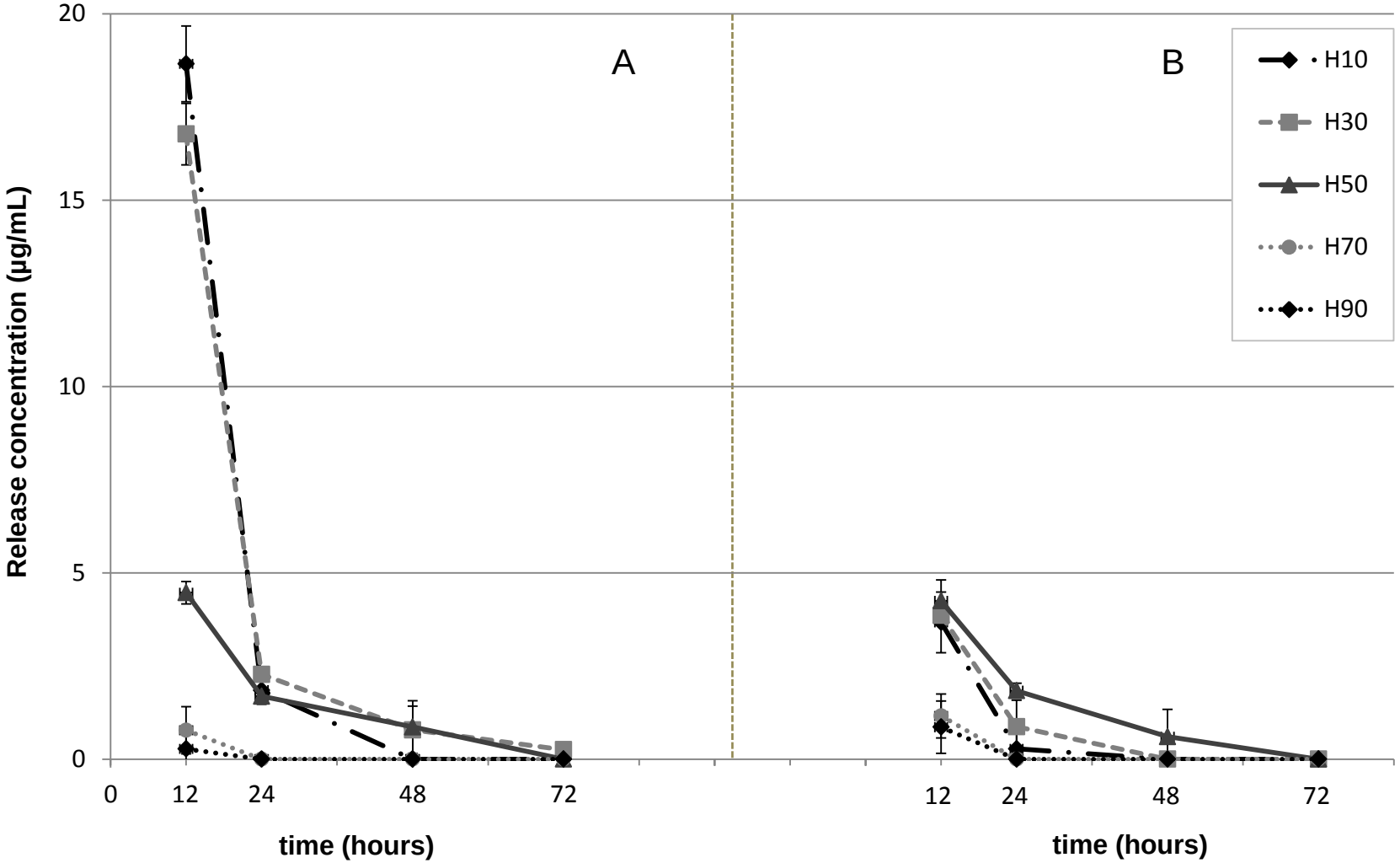


Fig. 4. Concentrations of CPC released into water from each hydrogel.
A: particles as prepared, B: washed particles. The bar represents the standard deviation of five replicates.

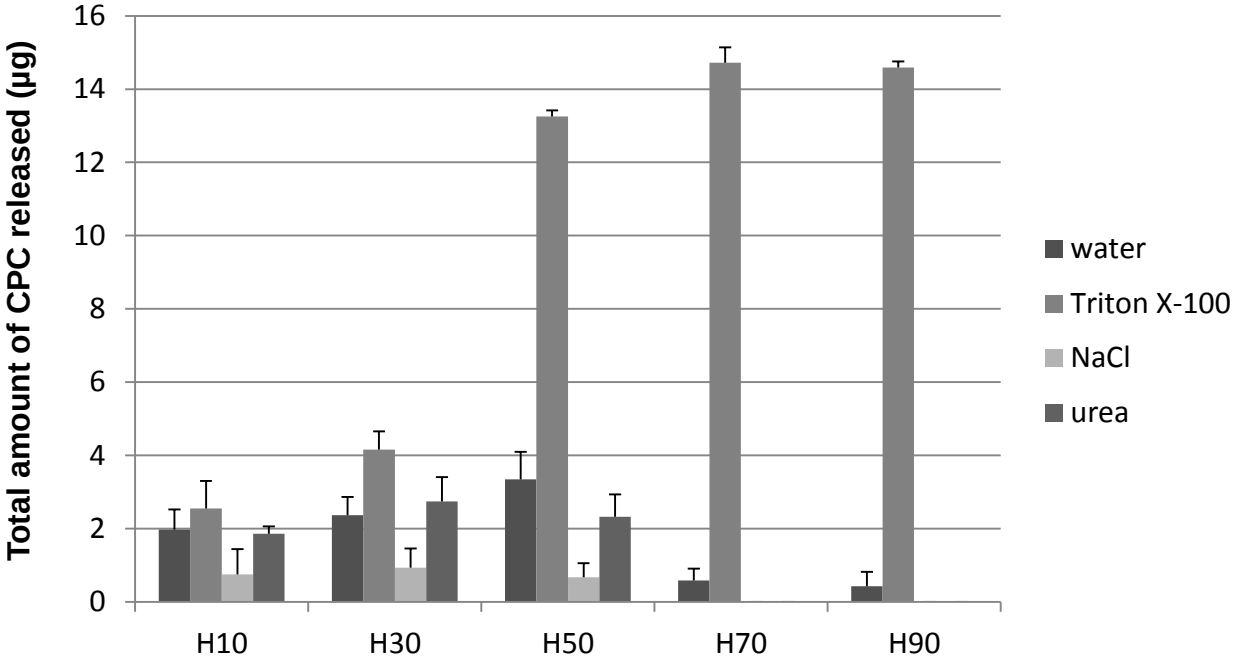


Fig. 5. Total amount of CPC released from hydrogel particles into water (control), Triton X-100, NaCl, or urea solutions.
The bar represents the standard deviation of five replicates.

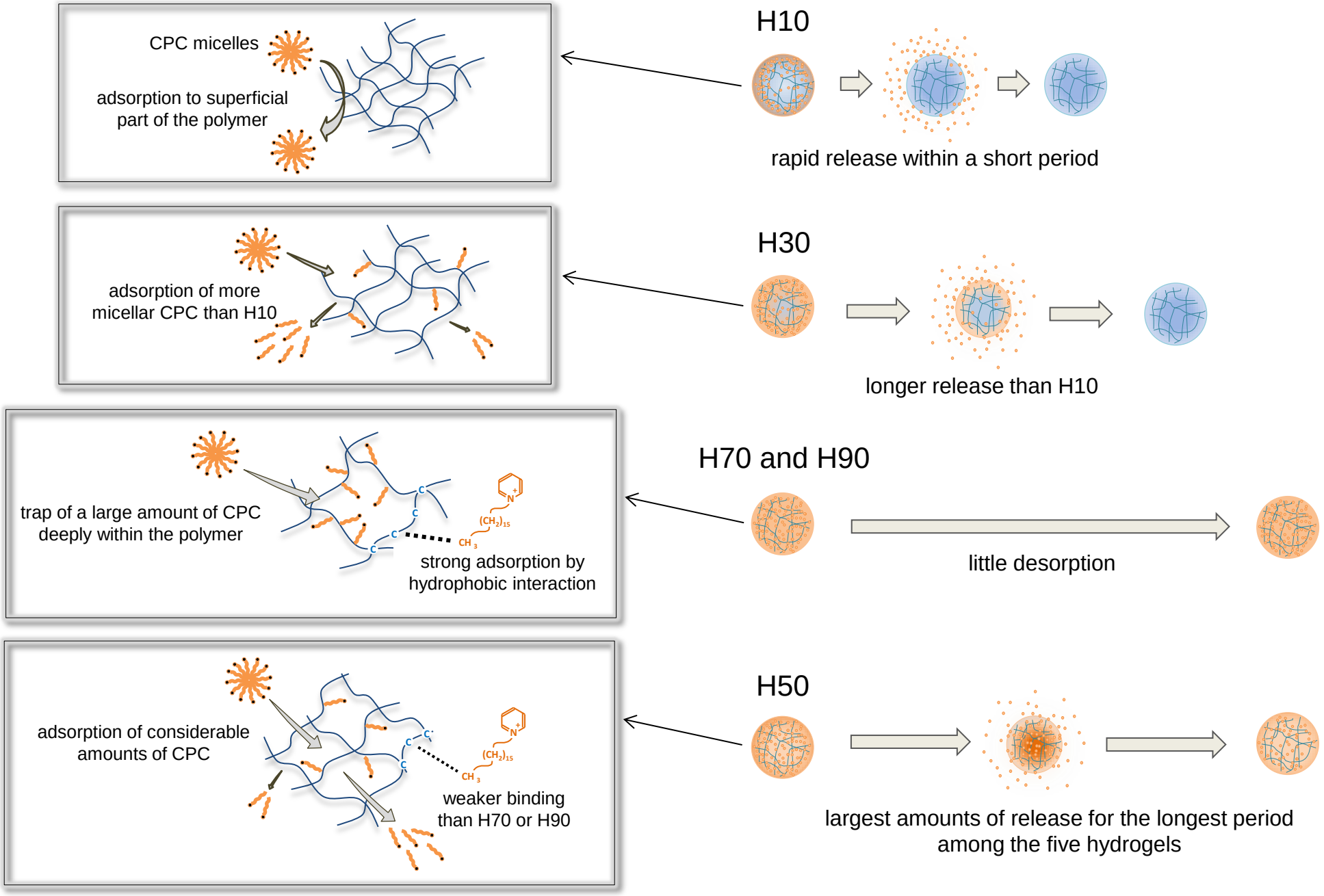


Fig. 6. Schematic diagram for loading of CPC and its release from poly-HEMA/TMPT hydrogels.

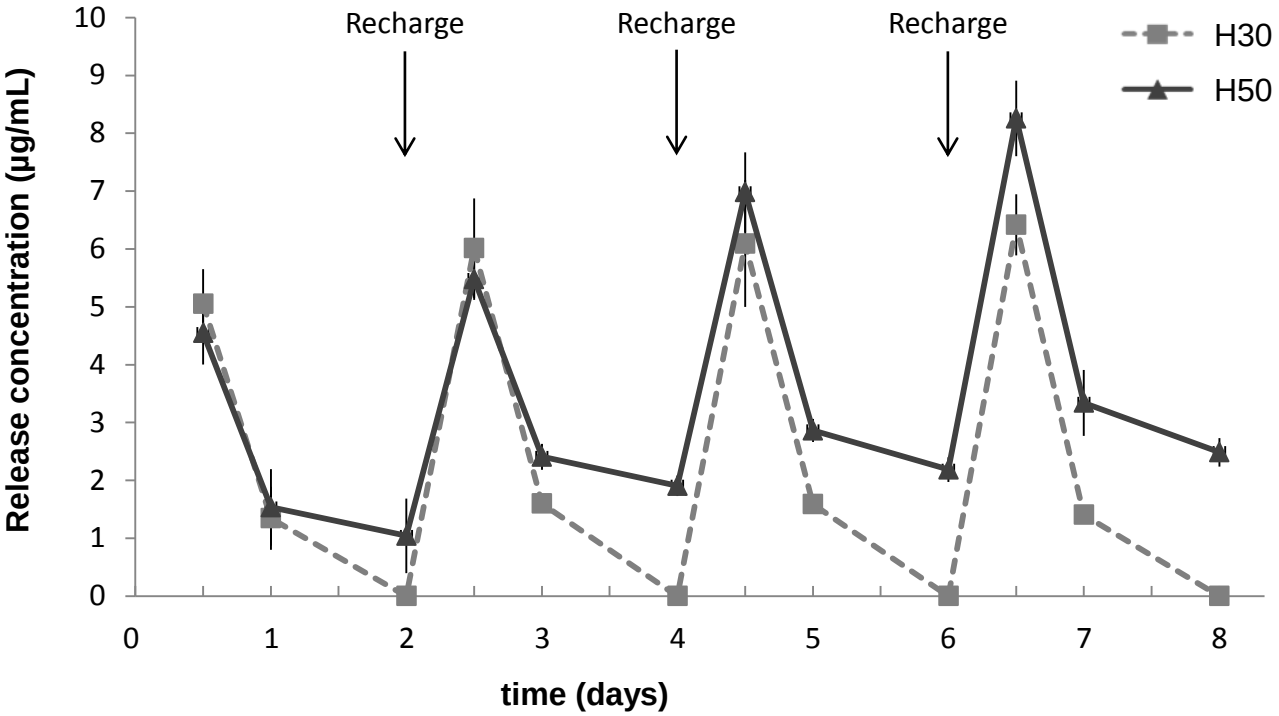


Fig. 7. CPC-release profile of H30 and H50 with recharging
The bar represents the standard deviation of five replicates**.**

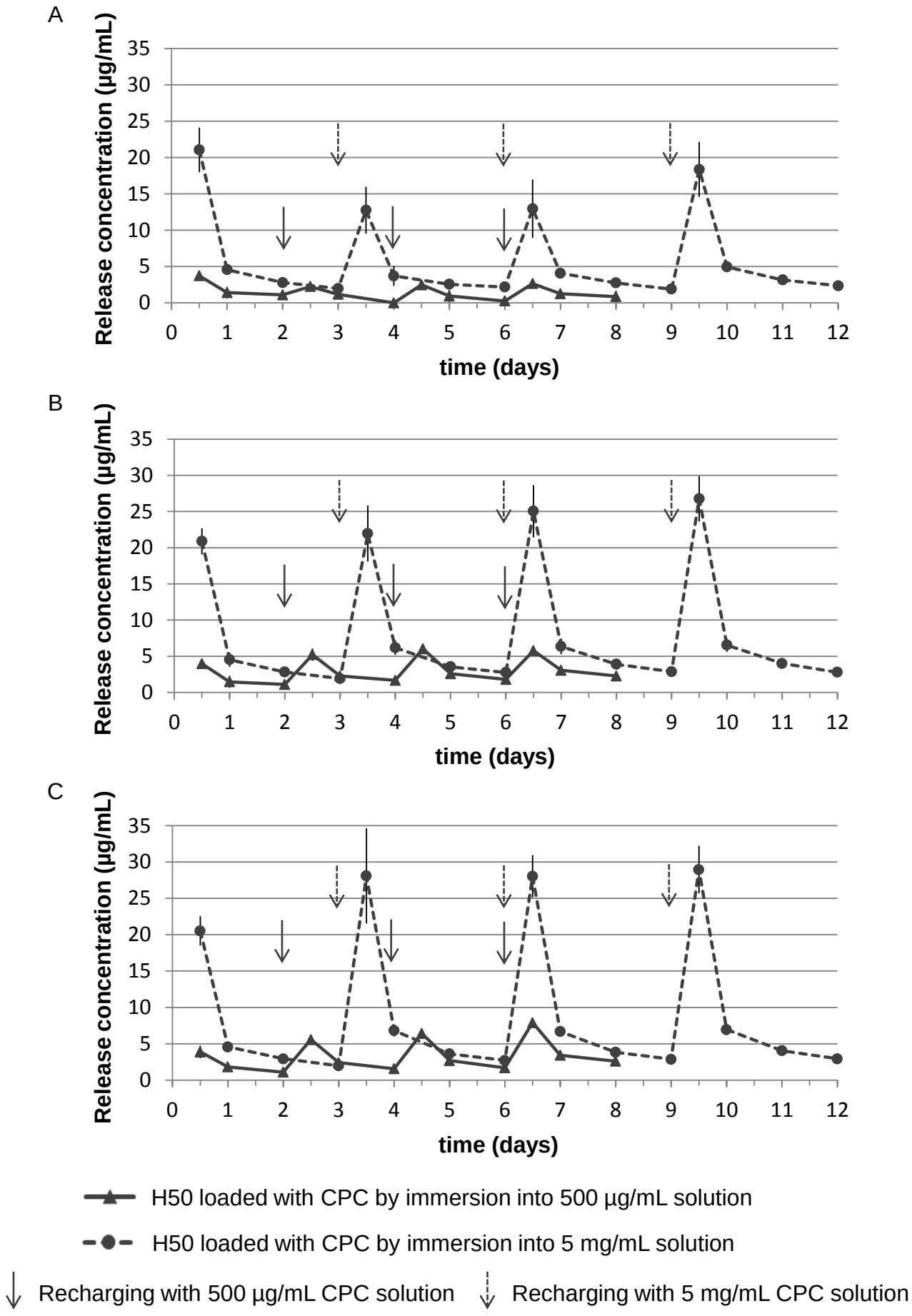


Fig. 8. CPC-release profiles for H50 under different recharging protocols. Recharging time A: 5 min, B: 6 h, C: 12 h. The bar represents the standard deviation of five replicates.

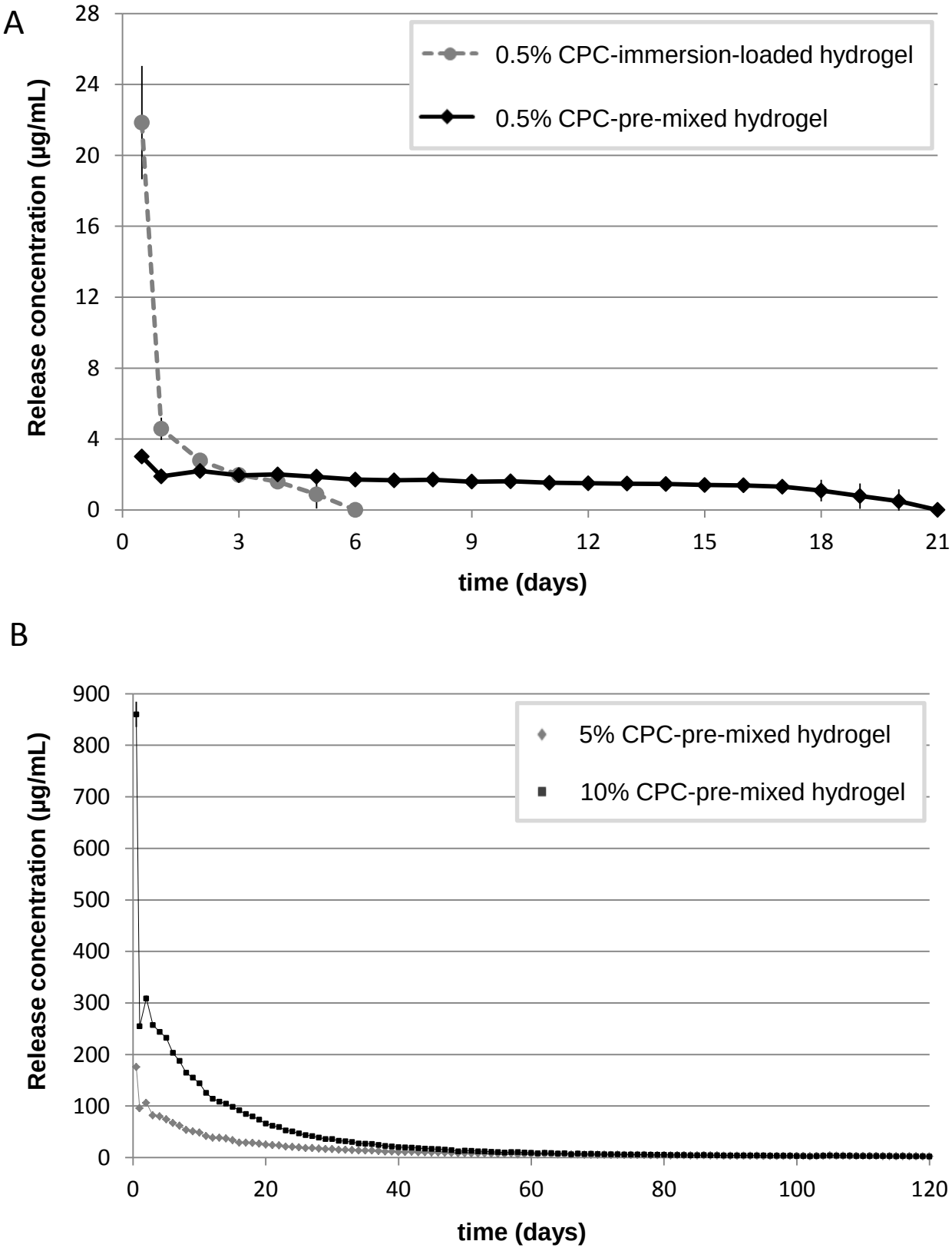


Fig. 9. CPC-release from hydrogels prepared by pre-mixing method.
A: 0.5% CPC-immersion-loaded and CPC-pre-mixed hydrogel, B: 5% and 10% CPC-pre-mixed hydrogels. The bar represents the standard deviation of five replicates.

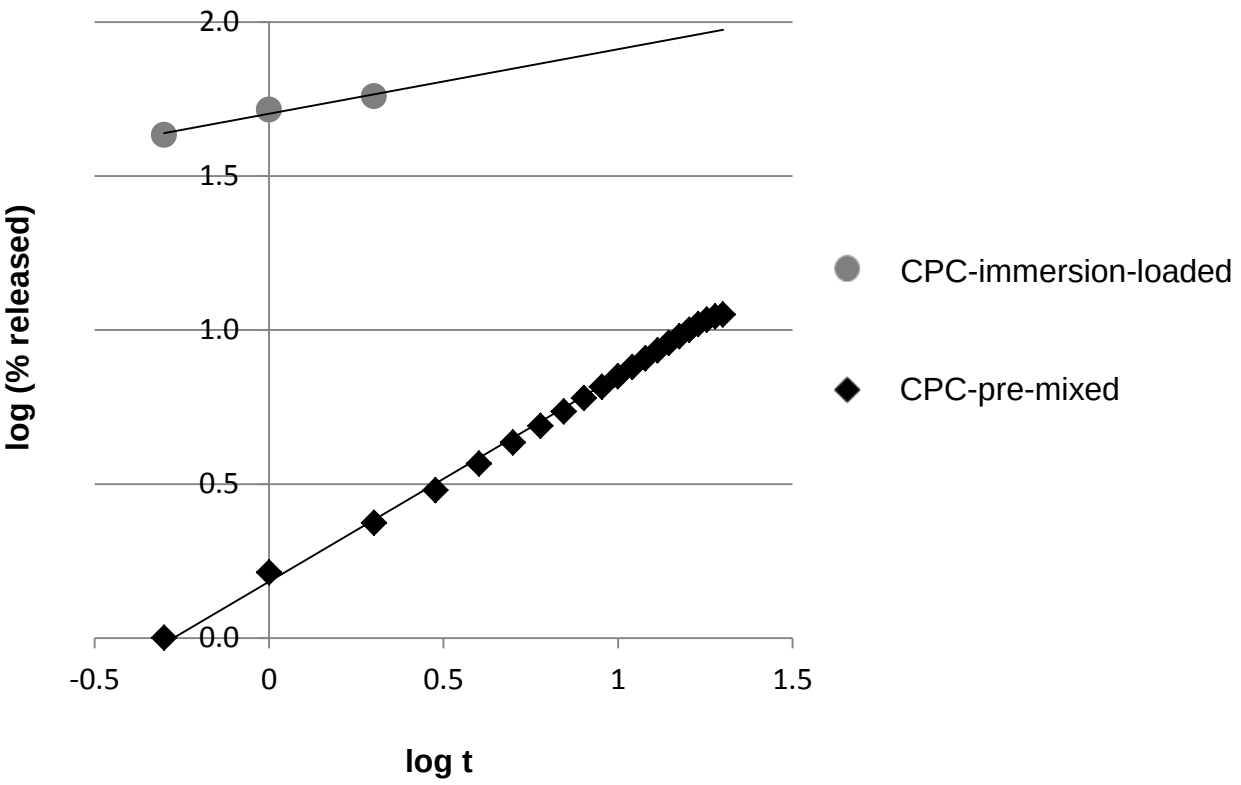


Fig. 10. The $\log_{10}$ fraction of total drug released from 0.5% CPC-immersion-loaded or pre-mixed hydrogels against $\log_{10}$ time.

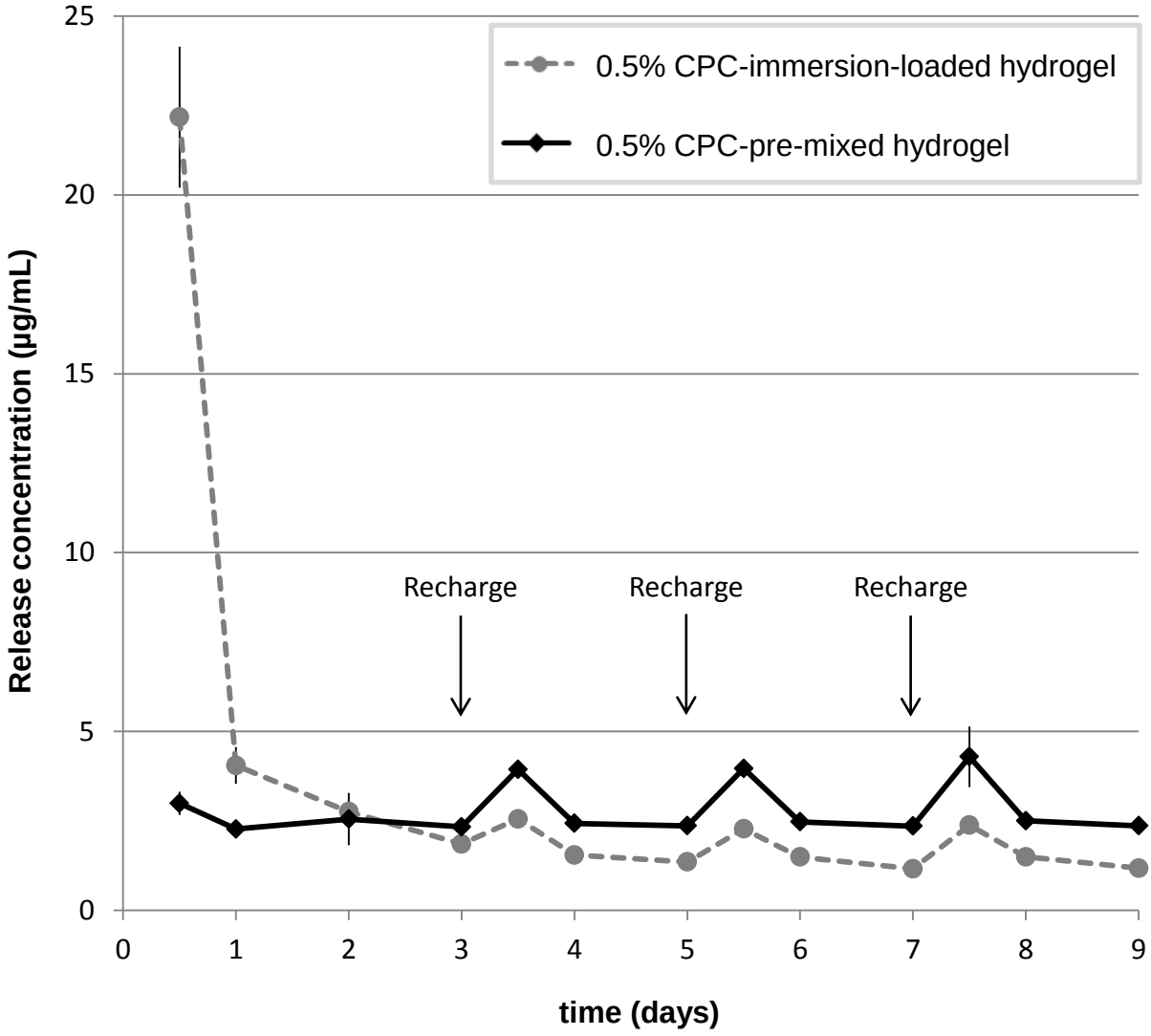


Fig. 11. CPC-release profile of 0.5% CPC-immersion-loaded and CPC-pre-mixed hydrogels with recharging.
The bar represents the standard deviation of five replicates.