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Complexation Hydrogels for the Oral Delivery of Growth Hormone and Salmon Calcitonin

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Abstract

The hydrogel system of poly(methacrylic acid-co-N-vinyl pyrrolidone) was evaluated for use as an oral delivery system for growth hormone and salmon calcitonin. These proteins were selected because of their therapeutic importance and the insight provided by evaluating the delivery of a therapeutic agent with a high molecular weight (growth hormone) and a drug with a high isoelectric point (salmon calcitonin). Growth hormone loading and release studies were performed for both P(MAA-co-NVP) and P(MAA-g-PEG). Loading efficiencies for the respective systems were 50.9 ± 1.8% and 57.8 ± 4.1%; weight incorporation of the protein was determined to be 3.5 ± 0.1% and 4.0 ± 0.3%. At pH 7.4, growth hormone release of 90% occurred within 45 min for P(MAA-co-NVP) microparticles; 90% release was not achieved with P(MAA-g-PEG) microparticles until 180 min. At pH 1.2, no release occurred from P(MAA-co-NVP) microparticles but 10% release occurred from P(MAA-g-PEG) microparticles. Salmon calcitonin loading and release were shown to be affected by the negative charges of deprotonated MAA; for systems with monomer molar feed ratios of 4:1, 1:1 and 1:4 MAA:NVP, loading efficiencies were determined to be 70.6 ± 3.0%, 25.3 ± 1.2%, and 1.6 ± 1.3%. Salmon calcitonin release was minimal from the copolymer with 4:1 MAA:NVP monomer feed at pH 7.4. The release improved when the pH was raised above physiological levels. These studies confirmed that P(MAA-co-NVP) was an effective oral delivery system for high molecular weight drugs, but improvements are needed before the system could be utilized for high isoelectric point therapeutic delivery.

Keywords

hydrogels; oral delivery; growth hormone; salmon calcitonin

Introduction

Controlled delivery of therapeutic agents continues to be a problem when these agents are of high molecular weight or exhibit high isoelectric points (pI > 7.0). Here, we discuss the release of two such agents utilizing a family of promising hydrogels.

Growth hormone, or somatotropin, is a single polypeptide chain consisting of 191 amino acids. The molecular weight varies based on the species of origin: human, 22124 Da;

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bovine, 22256 Da. The isoelectric point of growth hormone is 4.9[1] and the chain has a hydrodynamic radius of approximately 24-25 Å[2,3]. The protein is synthesized in and released from somatotrophs[4], a cell type located in the anterior pituitary gland at the base of the brain.

Growth hormone deficiency is an abnormality occurring in 20,000 children and approximately 50,000 adults in the United States[5]. It is marked by a lack of secretion by the pituitary gland, resulting in hyposomatotropism. One possible reason for this is a hypothalamic problem leading to insufficient or abnormal synthesis of GHRH. The result of growth hormone deficiency is a decelerated growth rate or growth failure[6]. Furthermore, in adults, certain cardiovascular risk factors are heightened, including an increase in fat content and insulin resistance[5]. The deficiency can be treated by supplementary administration of recombinant human growth hormone (rhGH).

Calcitonin is a 32 amino acid single chain peptide hormone produced in the parafollicular cells, or C cells, of the thyroid gland[7]. In non-mammalian species, the hormone is secreted from the ultimobranchial gland[8]. Calcitonin has a molecular weight of 3432 Da[9] and is marked by a high isoelectric point of 8.86[10]. Three classes of calcitonin have been identified and each has a different level of potency to inhibit bone resorption. In order of highest to lowest potency, they are teleost/avian, artiodactyl, and primate. The non-mammalian calcitonins have more biological potency because of their more basic structure. It is for this reason that salmon calcitonin, part of the teleost/avian classification, is often used in mammals[11].

Osteoporosis and Paget's disease are two of the most prevalent diseases for which calcitonin is used as a treatment. It is also used in the treatment of hypercalcaemia and bone metastases. Osteoporosis is marked by low bone density and deterioration of the tissue within the bone, resulting in increasing fragility[12]. An estimated 28 million women in the United States have either osteoporosis or are at risk to be diagnosed with the disease due to low bone mass[13]. Women are the primary victims of osteoporosis due to the onset of bone mass decrease when estrogen levels are lower post-menopause, but men are affected by the disease as well. Calcitonin is used in osteoporotic patients to prevent bone loss and increase bone mineral density (BMD). It has been shown that the drug is also able to reduce the risk of fracture as BMD decreases[14] and provide an analgesic, pain-relieving effect[15,16].

For both growth hormone and salmon calcitonin, the primary administration option is injection therapy. An improved delivery option would be the oral route, an option that eliminates the fear[17] and pain[18] associated with injection therapy and improves the quality of life of the patient. Stimuli-sensitive hydrogels are a class of carriers that have been rigorously evaluated for site-specific drug delivery; a thorough review was prepared by Peppas et al.[19]. With the oral delivery of proteins, the site of interest is the upper small intestine and the dominant stimulus in the transition between the stomach and small intestine is pH. pH-sensitive gels, therefore, have been investigated for use in oral delivery applications. These gels can be neutral, ampholytic, cationic or anionic[20]. Cationic gels are swollen at a pH below the pK_a of the ionic constituent and deswell above the pK_a; anionic gels operate in the reverse manner and are swollen at conditions above the pK_a. Since the pH transition in the GI tract is from low to high, anionic gels provide the best option for a pH-sensitive hydrogel carrier for the oral delivery of proteins.

It is well known that hydrogels are three-dimensional matrices that provide a location for drugs to be loaded and shielded until the appropriate time of release. In addition to the chemical crosslinks found in hydrogel systems, physical crosslinks in the form of complexes can form between constituents in copolymer or multipolymer systems. When using

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polyacids like poly(methacrylic acid) or poly(acrylic acid), these complexes take the form of hydrogen bonding complexes between polyacid and nonionic constituents[21]. Interpolymer complexes that are stabilized by hydrogen bonds form between electron deficient groups like polyacids, and electron dense groups like ethers and pyrrolidones[22]. The presence of these hydrogen bonds provides further protection to the loaded protein. An anionic gel with hydrogen bonding complexes would be in a complexed state below the pK_a of the ionic constituents; above the pK_a, the complexes break due to deprotonation of the polyacid pendant groups, leading to electrostatic repulsion and swelling of the gel[23].

The hydrogel system of methacrylic acid (MAA) and poly(ethylene glycol) (PEG) has been rigorously evaluated for oral delivery purposes[24-27]. The system possesses hydrogen bonding complexes between PEG and MAA[24] but the presence of PEG in the hydrogel has been shown to delay protein release upon reaching intestinal pH levels[25] and not fully prevent drug release in gastric conditions[26,27]. In this work, a system of MAA and N-vinyl pyrrolidone (NVP) was prepared for the oral delivery of growth hormone and salmon calcitonin. The monomer NVP was selected because in previous studies in our laboratories we pointed out that it provides an improved balance of hydrophilic/hydrophobic interactions and (possibly) also improved hydrogen-bonding [10,22]. The loading and release capabilities of the systems were investigated to determine if this hydrogel improved upon the limitations of the P(MAA-g-PEG) system.

Materials and Methods

Hydrogel Synthesis

P(MAA-co-NVP) hydrogels were synthesized using a UV-initiated free radical polymerization. For P(MAA-co-NVP), the monomer mixture included methacrylic acid (MAA) (Sigma-Aldrich, St. Louis, MO) and N-vinyl pyrrolidone (NVP) (Sigma-Aldrich). Ethylene glycol dimethacrylate (EGDMA) (Acros Organics, Morris Plains, NJ) was used as the crosslinking agent. All components were used as received.

The molar feed ratios of MAA:NVP for the copolymers synthesized were 1:1, 4:1, and 1:4. The crosslinking ratio was 1.0 mol % of the total monomer content. Irgacure® 184 (1-hydroxycyclohexyl phenyl ketone, Sigma-Aldrich) photo-initiator was added in the amount of 1.0 wt % of the total monomer content. The solvent makeup consisted of a 50:50 w/w water and ethanol mixture and was added to yield a 50:50 w/w total monomer to solvent ratio.

The components were prepared in an amber bottle and briefly sonicated (Bransonic® 8510, Branson Ultrasonics Corp., Danbury, CT) to yield homogeneity. The mixture was placed in a sealed glove box and bubbled with nitrogen for 20 min to remove oxygen from the environment. The mixture was then pipetted between two glass plates (150 × 150 mm^2) separated by a Teflon spacer (0.7 mm thick) and exposed to UV light (Dymax 2000 Light Curing System, Torrington, CT) at an intensity of 16-17 mW/cm^2 for 30 min to yield the polymer film. Following synthesis, the film was removed from the glass plates and washed with deionized water (changed daily) for 7 days to remove unreacted components. The washed film was vacuum dried at 30°C for 48 hours and crushed using a mortar and pestle. Sieves were used to yield particles in the size range of 90-150 μm. The particles were stored in a desiccator until use.

Growth Hormone Loading

A stock solution of growth hormone (somatotropin from porcine pituitaries, Sigma-Aldrich, St. Louis, MO) was prepared at a concentration of 0.5 mg/mL. 10 mg of growth hormone was added to 16 mL 1× PBS that had been acidified by the addition of 4 mL 0.1N HCl.

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Following dissolution, the pH was returned to 7.4 by the addition of 4 mL 0.1N NaOH. Sigmacote (Sigma-Aldrich) was used on all glassware to prevent particle and protein adsorption during loading.

140 mg of P(MAA-co-NVP) microparticles were added to 20 mL stock solution and allowed to equilibrate for 6h. After the growth hormone was allowed sufficient time to partition into the microparticles, 10 mL 0.1N HCl was added to the solution to lower the pH and collapse the microparticles. The collapsed microparticles were collected by filtration via water aspiration. The particles were washed with 10 mL deionized water, collected into a 50 mL centrifuge tube and frozen at -80°C. The frozen particles were lyophilized to yield a dry powder (Labconco FreeZone 4.5 Liter Benchtop Freeze Dry System, Kansas City, MO). The prepared particles were placed in a desiccator and stored at -20°C until needed.

For comparative purposes, P(MAA-g-PEG) 1:1 1% EGDMA microparticles (size range: 90-150 μm) were also prepared using an identical synthesis procedure to that of P(MAA-co-NVP) microparticles. The growth hormone loading procedure covered in this section was performed on the P(MAA-g-PEG) microparticles as well.

To determine the amount of protein loaded into the particles, four samples were drawn during the course of the procedure. The first was prior to the addition of particles to the stock solution; the second was following loading but prior to particle collapse; the third was after the particles were collapsed; and the final was from the filtration collection flask following particle washing. For each sample point, 0.2 mL were drawn and collected in HPLC vials (300 μL polypropylene screw neck vials with Lectrabond cap and PTFE/silicone septa, Waters Corp., Milford, MA) following filtration with a 0.22 μm PVDF filter (Millipore Millex-GV filter unit, Bedford, MA). HPLC analysis was performed on a Waters 2695 Alliance separations module (Waters Corp., Milford, MA) equipped with a Waters 2487 Dual λ Absorbance detector (Waters Corp.) with a Symmetry300™ C4 column (particle size = 5 μm; dimensions = 3.9 mm ID × 150 mm length, Waters Corp.). The mobile phase was 70% water (0.1% trifluoroacetic acid)/30% acetonitrile (0.08% trifluoroacetic acid) with a 20 μL injection volume.

Growth Hormone Release

Growth hormone release from the microparticles was evaluated by studies conducted using United States Pharmacopeia (USP) 28 Apparatus 2 (Distek Dissolution System 2100B; North Brunswick, NJ) with overhead impellers operating at 100 rpm and a bath temperature of 37°C.

For these studies, 15 mg of microparticles were added to a solution of 20 mL 1× PBS and 20 mg Tween 20 at pH 7.4 in Sigmacoted glass vessels. At time points over a 4h period, 0.2 mL were drawn from the release vessels and collected in HPLC vials following filtration with a 0.22 μm PVDF filter as described previously. Additional studies were also conducted with 15 mg of microparticles in 20 mL 0.1N HCl. Pre-warmed buffer was added to keep the volume in the vessels constant throughout the studies. The vials were analyzed via HPLC using the previously described method.

Salmon Calcitonin Loading

A stock solution of salmon calcitonin (Calbiochem, San Diego, CA) was prepared at a concentration of 0.2 mg/mL. 7 mg of salmon calcitonin were added to 28 mL 1× PBS that had been acidified by the addition of 3.5 mL 0.1N HCl. Following dissolution, the pH was returned to 7.4 by the addition of 3.5 mL 0.1N NaOH. Sigmacote (Sigma-Aldrich) was used on all glassware to prevent particle and protein adsorption during loading. Additionally, all

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glassware, utensils and buffers used for the loading of salmon calcitonin were autoclaved prior to use.

140 mg of P(MAA-co-NVP) microparticles were added to the stock solution and allowed to equilibrate for 3h. After the salmon calcitonin was allowed sufficient time to partition into the microparticles, 10 mL 0.1N HCl was added to the solution to lower the pH and collapse the microparticles. The collapsed microparticles were collected by filtration via water aspiration. The particles were washed with 5 mL deionized water, collected into a 50 mL centrifuge tube and frozen at -80°C. The frozen particles were lyophilized to yield a dry powder (Labconco FreeZone 4.5 Liter Benchtop Freeze Dry System, Kansas City, MO). The prepared particles were placed in a desiccator and stored at -20°C until needed.

Salmon Calcitonin Release

Salmon calcitonin release from the microparticles was evaluated by studies conducted using a United States Pharmacopeia (USP) 28 Apparatus 2 (Distek Dissolution System 2100B; North Brunswick, NJ) with overhead impellers operating at 100 rpm and a bath temperature of 37°C.

For these studies, 10 mg of microparticles were added to a solution of 12 mL 1× PBS at pH 7.4 in Sigmacoted glass vessels. Proof-of-concept studies were also conducted in alkaline borate buffer at pH 10.0. At time points over a 2h period, 0.2 mL were drawn from the release vessels and collected in HPLC vials following filtration with a 0.22 μm PVDF filter as described previously. Pre-warmed buffer was added to keep the volume in the vessels constant throughout the study. The vials were analyzed via HPLC using the previously described method.

Results And Discussion

Growth Hormone Loading

The loading efficiency for growth hormone and salmon calcitonin was calculated using the following relationship where C_o and C_f are the initial and final protein concentrations in solution (Equation (1)). The weight incorporation was calculated by Equation (2) where m_o is the initial mass of protein in solution, m_f is the mass remaining in solution following particle washing, and m_p is the mass of microparticles added to the stock solution for loading.

$$\text{Loading Efficiency} = \frac{C_o - C_f}{C_o} \times 100\% \quad (1)$$

$$\text{Weight Incorporation} = \frac{m_o - m_f}{m_o - m_f + m_p} \times 100\% \quad (2)$$

The loading of growth hormone into P(MAA-co-NVP) and P(MAA-g-PEG) formulations was successful. Testing this drug was a very relevant task because the results were not only important for the development of an oral delivery system for growth hormone, but they also provided insight into the ability of this system to work with other higher molecular weight drugs.

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These results show that for P(MAA-g-PEG)-based formulations, the loading efficiencies were 57.8 ± 4.1% while for (MAA-co-NVP)-based systems they were 50.9 ± 1.8%. These results were within statistical error of the method of determination of the growth hormone concentration. Figure 1 shows the weight incorporation which was higher in P(MAA-g-PEG) (4.1 ± 0.1%.) than in P(MAA-co-NVP)-based systems (3.5 ± 0.1%).

Growth Hormone Release

Studies were conducted to determine the stability of the protein under the conditions of loading and it was determined hat these formulations were stable. The comparative results of growth hormone release from P(MAA-co-NVP) and P(MAA-g-PEG) are shown in Figure 2. The amounts of growth hormone reported are discernable and can be measured with the techniques used here. The release of growth hormone from the P(MAA-co-NVP) microparticles occurred more rapidly and in a more controlled fashion than the release from the P(MAA-g-PEG) system. With the P(MAA-co-NVP) microparticles, more than 90% of the growth hormone originally in the network was released within 45 min at pH 7.4; it was not until 180 min that this value was obtained for the P(MAA-g-PEG) microparticles. At 3h, it is likely that the microparticles would have passed out of the upper small intestine, thus missing the targeted release location. The P(MAA-co-NVP) microparticles released the drug within a reasonable time window for upper small intestinal targeting and were a more favorable system for the oral delivery of this protein. Furthermore, no protein was released from the P(MAA-co-NVP) microparticles at pH 1.2. Growth hormone was clearly released from the P(MAA-g-PEG) system at low pH, exposing the protein to degradation within the stomach.

Salmon Calcitonin Loading

The greatest challenge with the loading of salmon calcitonin was the high isoelectric point. With an isoelectric point of 8.86[10], the drug was cationically charged at the pH for loading and release. As the carrier was anionic and covered with negative charges, the positively charged cationic drug was favorably attracted to the polymer and was not disposed to release from the network at the desirable time or pH.

As shown in Figure 3, the presence of negative charges was the dominating force for the loading of salmon calcitonin as the 4:1 MAA:NVP copolymer was capable of loading more drug than the other systems, with a 70.6 ± 3.0% loading efficiency and weight incorporation of 1.01 ± 0.04%. Although not shown, it is notable to report that the 4:1 system had 100% loading prior to the addition of HCl to collapse the microparticles, therefore the drug was completely inside the network due to the negative and positive charges. The 1:1 system had a loading efficiency of 25.3 ± 1.2% and weight incorporation of 0.35 ± 0.03%, whereas the 1:4 formulation had a loading efficiency of 1.6 ± 1.3% and weight incorporation of 0.03 ± 0.02%.

Characterizations of the 1:4 system not shown in this article revealed a high degree of swelling at low pH levels and fragility at high pH. It was speculated that this material would not be a strong candidate for drug delivery due to these properties and the results of the loading experiment confirmed this.

Salmon Calcitonin Release

Following confirmation of the poor loading ability of P(MAA-co-NVP) 1:4 1% EGDMA, it was decided to only test the salmon calcitonin release from the 4:1 and 1:1 systems. The release profiles may be seen in Figure 4. At pH 7.4, the 4:1 system released approximately 20% of the loaded drug within 10 min and sustained this amount through the release period, signifying that most of the protein remained inside the network. The 1:1 microparticles

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demonstrated release of approximately 33% of the loaded salmon calcitonin. The affinity of the drug to the carrier remained the dominant force during release and does not allow for this carrier to release a majority of the loaded contents.

To verify the importance of the charge attractions, a proof of concept release study was conducted at pH 10 in alkaline borate buffer. At pH 10, the carrier and the drug were both negatively charged as the pH was greater than the pI of salmon calcitonin. This change in pH did not significantly affect the release from the 1:1 microparticles, where release increased from 33% to approximately 40%. The 4:1 system, however, demonstrated an increase in salmon calcitonin release from 20% to approximately 65% release within 10 min. This confirms that charge repulsion was in place at this pH and the protein was ably released.

Conclusions

Growth hormone was loaded into P(MAA-co-NVP) and P(MAA-g-PEG) networks, and comparatively, more drug was able to load into the P(MAA-g-PEG) material. During release, the P(MAA-co-NVP) system demonstrated better release within the first hour and proved to be more suitable for drug delivery to the upper small intestine. Furthermore, no growth hormone release occurred from the P(MAA-co-NVP) microparticles in gastric conditions. These studies proved that the P(MAA-co-NVP) formulation could serve as a suitable carrier for the delivery of high molecular weight drugs.

Salmon calcitonin loading was greatly affected by the isoelectric point of the drug. At the pH of loading and release within the body, the protein was positively charged and remained attracted to the anionic charges of the polymer chains. As the number of negative charges in the system increased, more salmon calcitonin loaded in the system. As a proof of concept, the release of salmon calcitonin increased when the pH was raised beyond physiological levels and above the isoelectric point, proving that the charges are a dominating factor in the loading and release of therapeutic proteins.

Overall, the P(MAA-co-NVP) microparticles demonstrated great potential as a carrier for oral protein delivery of high molecular weight proteins. The prevention of drug release in acidic conditions was an improvement over other hydrogel based systems tested for oral delivery, and the novel material outperformed P(MAA-g-PEG) in release comparisons. Optimizations are needed to improve the ability of this system to effectively load and release drugs with high isoelectric points.

Acknowledgments

This contribution is dedicated to Professor Donald Paul on the occasion of his 70th birthday. Don has been a pioneer in diffusion in polymers and drug delivery since the very early of the field. His Paul-McSpadden model solved the Higuchi equation under exact boundary conditions and became a standard of design analysis of drug delivery systems. His pioneering work in hydrogels (1975-1985) paved the way for our understanding of diffusion in these important medical materials. NAP acknowledges with great pleasure a 35-year friendship with Don Paul.

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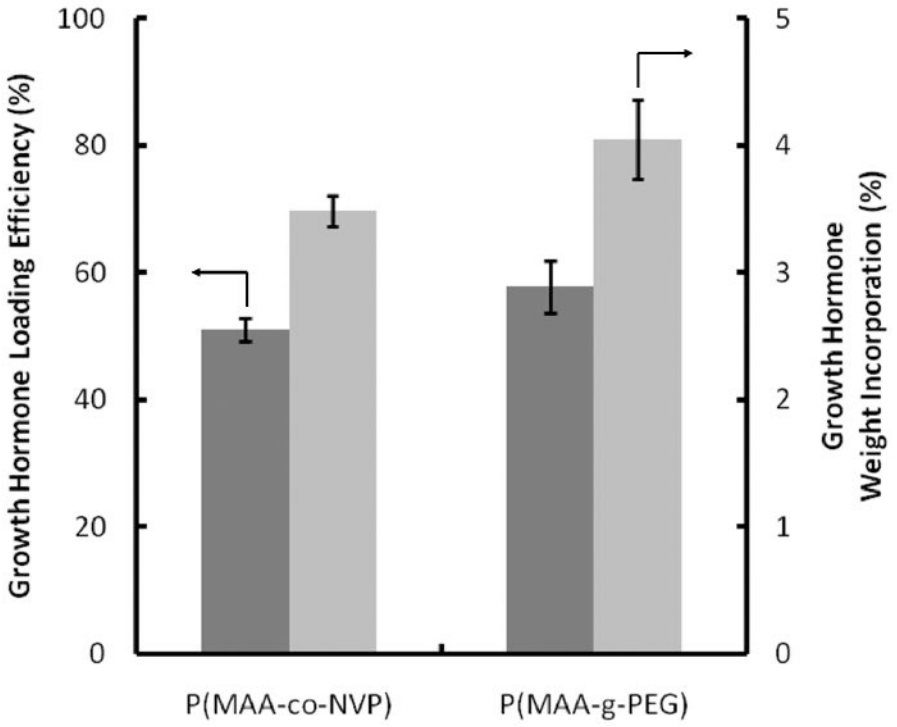

Figure 1.
Loading efficiency (left axis and columns) and weight loading efficiency (right axis and columns) of growth hormone in P(MAA-co-NVP) and P(MAA-g-PEG) microparticles, both 1:1 monomer molar feed amounts and 1% EGDMA crosslinking (90-150 μm particle size) (n=3 ± SD).

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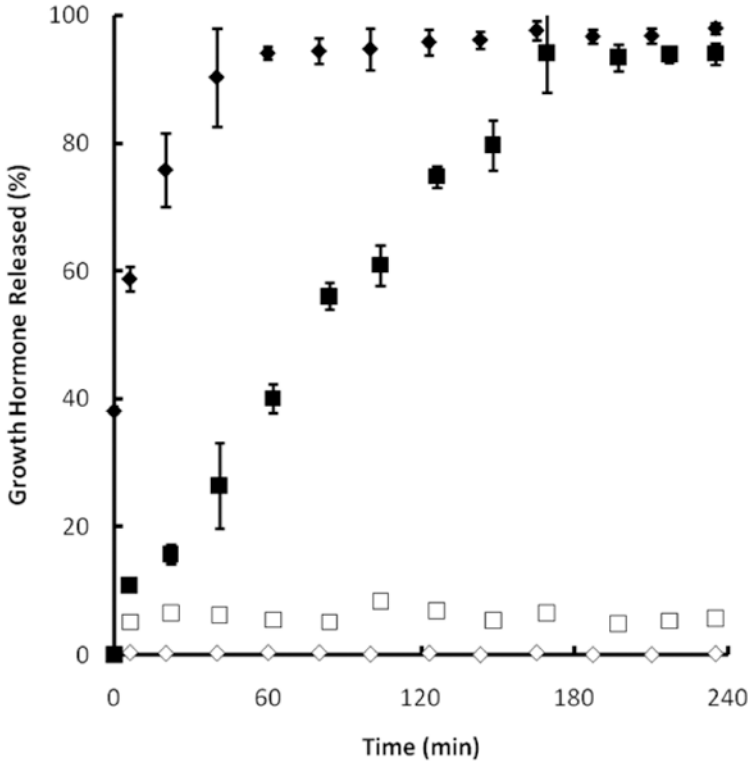

Figure 2.
Growth hormone release from two crosslinked polymer systems (90-150 μm particle size, both prepared from 1:1 monomer molar feed ratios and using 1% EGDMA as a crosslinking agent. Release was in 1× PBS and 0.1N HCl. 1× PBS. Polymer particle samples: P(MAA-co-NVP) (◆), P(MAA-g-PEG) (■) both in 0.1N HCl. Also polymer particle samples P(MAA-co-NVP) (◊), P(MAA-g-PEG) (□) in 1× PBS samples (n=3 ± SD); 0.1N HCl samples (n=1).

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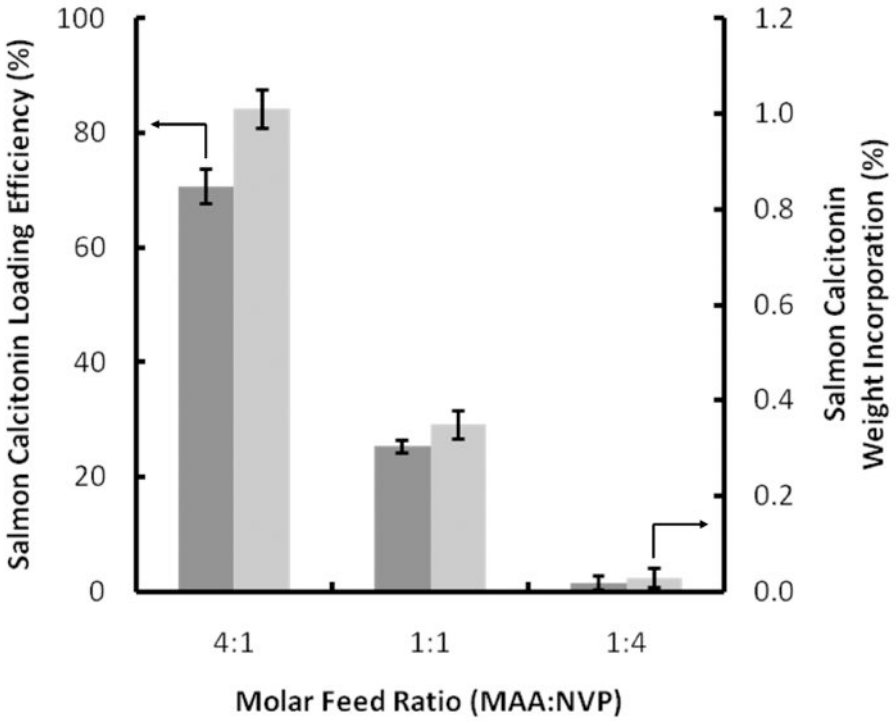

Figure 3.

Loading efficiency (left axis and columns) and weight loading efficiency (right axis and columns) of salmon calcitonin in P(MAA-co-NVP)-based microparticle (90-150 μm particle size) formulations. The particles were prepared with 1% EGDMA as a crosslinking agent at various MAA:NVP monomer molar feed ratios (n=3 ± SD).

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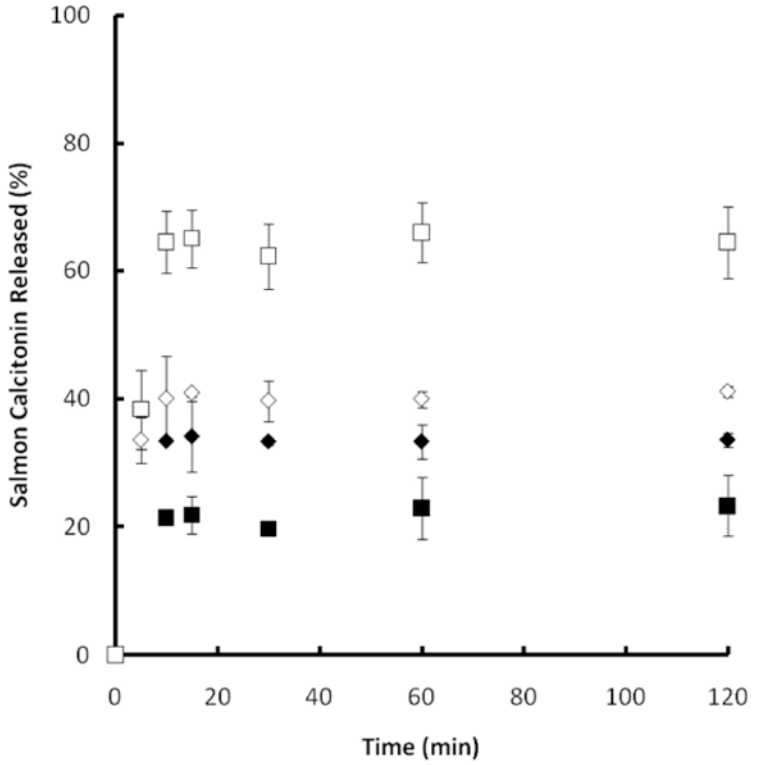

Figure 4.

Salmon calcitonin release from P(MAA-co-NVP)-based microparticle (90-150 μm particle size) formulations. The particles were prepared with 1% EGDMA as a crosslinking agent. Release was performed in 1× PBS (pH 7.4) and in an alkaline borate buffer (pH 10). Results are presented of release from formulations prepared with 1:1 monomer molar feed ratio and released in 1× PBS (◆) or in alkaline borate (◊). Results are also presented of release from formulations prepared with 4:1 monomer molar feed ratio and released in 1× PBS (■) or in alkaline borate (□) (n=3 ± SD).