Encapsulation of Opiorphin in Polymer-coated Alginate Beads for Controlled Delivery and Painkilling

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Abstract: Opiorphin (Oph) is a naturally produced endogenous peptide with a strong analgesic effect, superior to that of morphine, and without the severe side effects that morphine and morphine-like drugs exert. However, despite its strong therapeutic potential, the short duration of action, probably due to its low chemical stability and rapid degradation by the peptidases in the bloodstream, represents a serious obstacle to the Oph use into clinical practice. In this work a novel approach to construct Oph-loaded particles as a platform for its delivery has been developed. Gel beads loaded with Oph were synthesized from alginate, a naturally occurring biodegradable anionic polysaccharide, and coated with polyelectrolyte multilayers (from natural polyelectrolytes (chitosan and hyaluronic acid) and synthetic polyelectrolytes (poly(allylamine hydrochloride) and poly(styrene sulfonate)) or hybrid polyelectrolyte-graphene oxide multilayers. All coated Oph-loaded alginate beads show prolonged drug release compared to the non-coated ones, but the extent of the prolongation depends on the type of the coating. We expect that the successful encapsulation of opiorphin in biodegradable particles will provide an opportunity for the development of adequate drug delivery system with effective and prolonged analgesic activity and will offer a new alternative for pain management.

Keywords: Opiorphin, Alginate beads, Polyelectrolyte films, Graphene oxide.

Introduction
The treatment of acute and chronic pain, especially in terminally ill patients, is still an issue, despite the great efforts made in this regard at both scientific and economic levels. Problems of tolerance, risks of addiction and abuse, appearance of strong side effects still give rise to considerable restrictions to the use of opioids. Therefore, the search for new more effective therapeutic treatments against the pain is both of fundamental and economic importance.

Opiorphin (Oph) is a naturally produced short chain QRFSR-endogenous pentapeptide (H-Gln-Arg-Phe-Ser-Arg-OH) secreted into the human saliva [23], endowed with a strong analgesic and anti-depressant effect, even superior to that of morphine [20] and morphine-like drugs. However, despite the strong therapeutic potential of opiorphin, its short duration of
action, probably due to its low chemical stability and rapid degradation by the peptidases in the bloodstream, represents a serious obstacle to its use into clinical practice.

Administration of Oph (1 mg/kg) is reported to induce the same painkilling effect as 3 mg/kg morphine thus decreasing the paw-licking reflex in rats, and reducing the pain in the “pin-pain” test at a dosage six-times lower than that of morphine [17]. The exact mechanism of action of Oph is still to be clarified, even though a first structure-activity relationship study recently demonstrated that this peptide is a powerful dual inhibitor of the encephalin-inactivating Zn-dependent metallo-ectoendopeptidases neutral endopeptidase (NEP) and aminopeptidase N (AP-N) [16, 23]. These enzymes metabolize the opioid peptides, like enkephalins, endorphins and their derivatives that the human spinal cord creates in order to decrease the pain. Unfortunately, they break up quickly and have a short lifespan. By inhibiting the action of these enzymes, Oph slows down the natural break down of endorphins and they stay present for longer, bringing about the pain relief. Moreover, many of the side effects accompanying the morphine-like drugs should be absent, since Oph does not activate directly the opioid receptors. In fact, repeated intraperitoneal administration of Oph in mice, failed to produce tolerance or morphine-cross-tolerance phenomena as well as anti-peristaltic effects [17]. However, the rapid Oph degradation by the peptidases in the bloodstream and its short duration of action represent a serious obstacle to its application in the clinical practice [20]. A successful alternative strategy for enhancing Oph bioavailability could be the use of a suitable carrier.

The use of drug-loaded nano/microcapsules is a widely accepted approach to improve the efficiency of drug treatment and precisely deliver the bioactive compounds into specific body tissue or organ [13]. Up to now there is only one investigation reporting the entrapment of opiorphin in lipid (phosphatidylcholine/cholesterol/stearylamine) and lipid-poly(ethylene glycol) liposomes [12].

This study is focused on the development of a novel delivery platform for opiorphin based on alginate (Alg) beads. Alginate is a natural anionic polymer that has found numerous biomedical applications (tissue engineering and drug delivery) due to its biocompatibility, low toxicity and easy gelation by addition of divalent cations such as Ca$^{2+}$ [9, 21]. Sodium alginate is one of the most widely investigated polymers in the pharmaceutical and biomedical fields, because it is approved from U.S. Food and Drug Administration (FDA) and included into both the United States and the European Pharmacopeia [4, 19]. The alginate-based beads do not dissolve or swell in the stomach acids, but rapidly swell and disintegrate in the intestine which makes them preferred vesicle for delayed release of drugs. However, the porous alginate beads have low retention capacity for encapsulation of low molecular weight and water soluble drugs. Therefore, to prolong the degradation time and drug-release we coated the Alg-beads with polyelectrolyte multilayers (PEM) or hybrid PEM-graphene oxide. Two polyelectrolyte couples comprising natural (chitosan/hyaluronic acid, Chi/HA) and synthetic (poly(allylamine hydrochloride)/poly(styrene sulfonate), PAH/PSS) polyelectrolytes are employed and their effect on the release kinetics of the peptide was elucidated. The insertion of non-polymer component into the polymer matrix is an innovative strategy employed for modulating the properties of polyelectrolytes-based materials. Owing to its remarkable electrical, thermal, and mechanical properties, and two-dimensional structure graphene oxide (GO), modified with various polyelectrolytes (PE), is extensively studied as suitable delivery system of proteins, peptides, nucleic acid drugs, and chemotherapeutics, although its biocompatibility is still an issue [18]. GO has been shown to exert concentration-dependent cytotoxicity in A549 cell line that could be strongly attenuated by incubation with
10% fetal bovine serum, owing to its extremely high adsorption ability [8]. The aim of this study was to design suitable system for controlled delivery of the chemically unstable opiorphin that will prolong its circulation in the blood stream and its pharmacological activity.

**Materials and methods**

**Materials**

Sodium alginate was purchased from Carl Roth & Co. (Karlsruhe, Germany) and used as 1% (w/w) aqueous solution (viscosity of 1% solution at 20 °C: 350-550 mPas); fluorescein isothiocyanate labeled opiorphin, OphFITC (Seq: FITC-Acp-QRFSR) was obtained from NovoPro Bioscience Inc. (China). Low molecular weight chitosan (MW ≈ 50-190 kDa, 75-85% deacetylated) and poly(styrene sulfonate) (MW ≈ 70 kDa) were supplied from Sigma Aldrich (Steinheim, Germany); sodium hyaluronate (MW ≈ 360 kDa) was purchased from Contipro a.s. (Czech Rep.), poly(allylamine hydrochloride) (MW ≈ 120-200 kDa) was from Alfa Aesar & Co. (Karlsruhe, Germany). Graphene oxide is commercially available product purchased as water suspensions with a concentration of 4 mg/ml from Graphenea (Spain).

All polyelectrolytes (HA, Chi, PSS and PAH) were dissolved in 1% CaCl₂ to a concentration of 1 mg/ml (HA and Chi) or 2 mg/mL (PSS and PAH). The stock suspension of GO was diluted in ultra-pure water to the final concentration of 0.5 mg/ml and sonicated for 30 min in an ultrasonic bath (50 Hz, ISOLAB, Germany). 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) and N-hydrosulfosuccinimide (sulfo-NHS) (both from Sigma-Aldrich) were dissolved in 150 mM NaCl to concentration 50 mM.

**Preparation of Oph-loaded alginate particles**

OphFITC-loaded alginate beads were constructed following a protocol for preparation of alginate beads based on ionic cross-linking technique by dripping the solution of sodium alginate into 1% CaCl₂ solution used as a cross-linking agent [1, 7, 10, 14]. When a drop of sodium alginate solution faces Ca²⁺ ions, gelation immediately occurs onto its surface. Then Ca²⁺ ions diffuse inside the drop to induce gelation thus forming a particle. Particles were further left in the cross-linker solution overnight at 4 °C.

Sodium alginate was added to OphFITC water solution (1 mg/ml) to obtain an alginate final concentration of 1.25% (w/v). In all experiments the same volume (1 ml) of the OphFITC/Alg viscous mixture was added drop-wise to 5 ml of 1% CaCl₂ (w/v), pH 6.5. Thus prepared OphFITC-loaded Alg-beads were immediately transferred into the degradation medium.

**Coating of drug-loaded Alg-beads**

OphFITC-loaded Alg-beads were coated with PEM or hybrid PEM-GO coatings by application of Layer-by-Layer (LbL) technique proposed by Decher [5].

The as-prepared OphFITC-loaded Alg-beads (negatively charged) were first incubated in 5 ml of Chi or PAH solutions (positively charged PE) for 10 min under constant mild shaking, followed by triple washing in 1% CaCl₂ (each for 2 min). Next, the beads were incubated in 5 ml of HA or PSS (negatively charged PE) for 10 min and washed in 1% CaCl₂. This coating cycle was repeated until the desired number of polyelectrolyte layers was achieved. Graphene oxide was deposited as a substitute for the negatively charged polyelectrolytes at two different localizations in the PE matrix, followed by washing with ultra-pure water.
For chemical cross-linking of the Chi/HA multilayers, the Chi/HA-coated beads were kept into a freshly prepared equimolar mixture of EDC and sulfo-NHS overnight at 4 °C and then washed three times in 1% CaCl₂ (each for 1h).

**Drug release**
The kinetics of Oph\(^{FITC}\) release from bare and PEM-coated Alg-beads was followed by incubation in 10 ml of phosphate buffered saline (PBS) with pH 7.2 at room temperature under mild orbital shaking. At predefined time points the incubation medium was analyzed spectrophotometrically (BIO-SPEC NANO, Shimadzu). The absorbance of Oph\(^{FITC}\) at 496 nm was registered. The drug-loaded Alg-beads were further incubated in an equivalent volume of fresh PBS. The released amount of Oph\(^{FITC}\) was determined as a percentage of the total amount entrapped Oph\(^{FITC}\) and quantified as follows:

\[
\text{release(\%)} = \frac{\text{released Oph}^{FITC}}{\text{total Oph}^{FITC}} \times 100, \tag{1}
\]

where the numerator indicates the measured amount of released Oph, while the denominator indicates the total amount of the encapsulated peptide.

All experiments were performed in triplicate.

**Results and discussion**
Oph\(^{FITC}\) was first entrapped in biocompatible and biodegradable alginate hydrogel beads that were further coated with different PE or PE-GO coatings. The following Oph\(^{FITC}\)-loaded particles were fabricated and compared:

1) uncoated Oph-loaded Alg-beads;
2) Oph-loaded Alg-beads coated with weak natural PE without or with one surface GO-layer – (Chi/HA)\(_{2.5}\) and (Chi/HA)\(_{2.5}\)GO;
3) Oph-loaded Alg-beads coated with weak natural cross-linked PE without or with one surface GO-layer – (Chi/HA)\(_{2.5}\)(CL) and (Chi/HA)\(_{2.5}\)GO(CL);
4) Oph-loaded Alg-beads coated with strong synthetic PE without or with one surface GO-layer – (PAH/PSS)\(_{2.5}\) and (PAH/PSS)\(_{2.5}\)GO.

**Entrapment efficiency**
All experiments were performed with 1 mg initial amount of Oph. However, during the encapsulation into the Alg-beads and the coating with PE and PE-GO multilayers part of this amount was lost. Table 1 represents the amount of Oph\(^{FITC}\) entrapped into the different types of particles.

<table>
<thead>
<tr>
<th>Type of coating</th>
<th>Mean amount of entrapped Oph(^{FITC}) (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>uncoated</td>
<td>0.71 ± 0.09</td>
</tr>
<tr>
<td>(Chi/HA)(_{2.5})</td>
<td>0.68 ± 0.10</td>
</tr>
<tr>
<td>(Chi/HA)(_{2.5})GO</td>
<td>0.76 ± 0.11</td>
</tr>
<tr>
<td>(Chi/HA)(_{2.5})(CL)</td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td>(Chi/HA)(_{2.5})GO(CL)</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>(PAH/PSS)(_{2.5})</td>
<td>0.95 ± 0.10</td>
</tr>
<tr>
<td>(PAH/PSS)(_{2.5})GO</td>
<td>0.99 ± 0.05</td>
</tr>
</tbody>
</table>
Analysis of microbeads shaped calcium alginate gels by electron microscopy has shown that the polymer matrices have porous surface [2]. It is known that the encapsulation efficiency of low molecular weight drugs is low [3, 15] because of the high drug loss during preparation, which is in turn due to the fast diffusion of this type of drugs.

Data in Table 1 show that during Alg gelation in 1% CaCl₂ solution around 30% of the initial Oph-amount is lost. However, when the Alg gelation takes place in PE solution with 1% CaCl₂ the particles coated with weak natural PE lose 40-50% of the initial Oph-amount while those coated with strong synthetic PE demonstrate almost 100% encapsulation. There is almost no leakage of Oph from the particles covered with strong synthetic polyelectrolytes (PAH/PSS). Characteristic feature of strong synthetic polyelectrolytes is the high charge density, which allows strong electrostatic interaction with other charged molecules (for example negatively charged Alg-chains). The strong interaction between PAH and Alg perhaps creates immediately an Alg-PAH barrier on the particles' surface that hinders Oph molecules to freely diffuse out of the particles. The particles coated with weak polyelectrolytes (Chi/HA) show approximately the same encapsulated Oph-amount despite of the enhanced structural integrity of these coatings. The probable reason for the high level of Oph leakage is the longer protocol for coating and cross-linking (13.5 hours) compared to that for particles without cross-linking that required only about 1.5 hours for successful coating.

**Oph release**
The effectiveness of the developed PE and hybrid PE-GO coatings for improving and extending the analgesic effect of the OphFITC-loaded gel beads was investigated by incubation in physiological medium at constant mild shaking. Fig. 1 demonstrates the averaged release curves (n = 3) of OphFITC-loaded Alg-beads with different multilayer coatings, compared with uncoated control particles.

**Fig. 1** Oph release curves of uncoated Alg-beads (A, B, C) and Alg-beads coated with natural polyelectrolytes (Chi/HA)2.5 and (Chi/HA)2.5GO (A); with cross-linked natural polyelectrolytes (Chi/HA)2.5(CL) and (Chi/HA)2.5GO(CL) (B); and with synthetic polyelectrolytes (PAH/PSS)2.5 and (PAH/PSS)2.5GO (C).

The pharmacological potential of the coated and uncoated OphFITC-loaded Alg-beads was evaluated based on the OphFITC release curves and the relative pharmacological activity (RPA), i.e., the time interval during which the particles release more than 80% of the Oph content (Fig. 2, Table 2). The longer particular vehicle delivers Oph the higher the value of the corresponding RPA.
The Oph release curve of uncoated Alg-particles has an exponential decay profile – the maximum amount of the drug was released during the first 15 minutes of incubation in PBS and ca. 80% of the loaded Oph had left the particles at the end of the first hour. The RPA of the control particles is about 1 hour (Table 2, Fig. 2A). LbL-deposition of thin barrier polymer films on the Alg-particles affects significantly the profile of the release curves (Fig. 1). In case of both Chi/HA and PAH/PSS thin coatings the maximum of the release was shifted by approximately 1 hour and RPA was prolonged by about an hour compared to the uncoated particles (Fig. 2A and C, Table 2). The shift in the maximum of drug release and the prolongation of RPA of the coated particles is an indication that the presence of a thin polymeric shell creates a diffusion barrier which hinders the release of the Oph. During the first 30 to 60 minutes, the thin polymer shell becomes destabilized, which weakened its integrity and increased its permeability.

![Image](Fig. 2 Averaged cumulative percent of OphFITC released from uncoated Alg-beads (A, B, C); and Alg-beads coated with natural polyelectrolytes (Chi/HA)2.5 (B); cross-linked natural polyelectrolytes (Chi/HA)2.5(CL) and (Chi/HA)2.5GO(CL) (C); and with synthetic polyelectrolytes (PAH/PSS)2.5 (A) and (PAH/PSS)2.5GO (C).)

The cross-linking of Chi/HA layers exerts a significant effect on Oph release kinetics. The results show that the maximum of release and RPA were prolonged by about half an hour compared to the particles with non-crosslinked coatings and the stability of the coated Alg-beads is extended from 5 to 21 days (Fig. 1B and 2B, Table 2).

Table 2. Releasing parameters (time to reach maximum release and RPA) and structural stability of the OphFITC-loaded Alg-particles with different multilayer coatings.

<table>
<thead>
<tr>
<th>Type of coatings</th>
<th>Time to reach maximum release of Oph, [hours]</th>
<th>RPA, [hours]</th>
<th>Beads stability, [hours]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated</td>
<td>0.25</td>
<td>1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>(Chi/HA)2.5</td>
<td>1</td>
<td>1.8</td>
<td>5</td>
</tr>
<tr>
<td>(Chi/HA)2.5GO</td>
<td>1</td>
<td>1.5</td>
<td>5</td>
</tr>
<tr>
<td>(Chi/HA)2.5(CL)</td>
<td>1–1.5</td>
<td>2.2</td>
<td>21</td>
</tr>
<tr>
<td>(Chi/HA)2.5GO(CL)</td>
<td>2–2.5</td>
<td>3.2</td>
<td>*</td>
</tr>
<tr>
<td>(PAH/PSS)2.5</td>
<td>1–1.5</td>
<td>1.9</td>
<td>5</td>
</tr>
<tr>
<td>(PAH/PSS)2.5GO</td>
<td>1.5–2</td>
<td>2.8</td>
<td>*</td>
</tr>
</tbody>
</table>

* Particles that maintain the integrity for days

The profile of OphFITC-release from Alg-particles coated with the synthetic PAH/PSS polyelectrolytes is similar to that of the Alg-particles with cross-linked Chi/HA coatings – comparable RPA’s and maximal OphFITC-release after 1.5 hours of incubation. The similar
release kinetics of the two types of coated particles results from the covalent cross-linking between the weekly interacting Chi and HA chains which significantly improves the mechanical stability of Chi/HA multilayers and increase by 60% their resistance to degradation [6], thus resembling the properties of PAH/PSS films composed of strongly interacting synthetic polyelectrolytes.

The deposition of GO as a surface layer on top of the polymer matrix exerts a significant effect on the OphFITC-release from Alg-beads coated with (Chi/HA)2.5GO(CL) and (PAH/PSS)2.5GO coatings (Fig. 1). GO-sheets shape an additional diffusion barrier that enhances the structural stability of the particles with natural cross-linked Chi/HA and synthetic PAH/PSS coatings and significantly prolongs the RPA (up to ca. 3.2 hours) of the OphFITC-loaded particles (Table 2). The particles coated with hybrid PE-GO coatings release the peptide in smaller portions but for a longer time (Fig. 1B and 1C). On the other hand, the deposition of GO-layer has a destabilizing effect on the non-crosslinked Chi/HA system (Fig. 1) reducing the RPA by about 20 minutes.

The divergent effect of GO could be due to the altered mechanical properties of the thin films. In the case of cross-linked coatings and the ones built-up of synthetic polymers, characterized by smoother and more rigid surface, the GO nanosheets form nanocomposite complexes that further increase the mechanical stability and reduces the permeability of the Alg-particles. On the other hand, the non-crosslinked Chi/HA coatings are known to be highly hydrated and soft, with loose gel-like texture, and the interaction between the Chi and HA chains are weak and diffuser. The destabilizing effect of GO in this case could be due to the GO-induced detachment of a part of the surface adsorbed Chi-chains.

Another observation concerns the structural stability of the coated OphFITC-loaded Alg-beads along the incubation in PBS buffer. Table 2 represents the relative particles lifetime during the incubation period. Data show that the uncoated beads degrade much faster (for 4.5-5.0 hours) compared to the particles coated with the cross-linked natural Chi/HA/GO or synthetic PAH/PSS/GO thin hybrid films that retain their integrity for days. Generally, degradation of a Ca2+-crosslinked Alg-gel occurs by removal of the Ca2+ ions that destabilize and disintegrate the polymer matrix. This leads to leakage of the entrapped peptide and solubilization of the high molecular weight Alg-chains. Degradation of the Alg gel matrix can be prevented by storing the Alg-beads in a medium containing free Ca2+ ions and by maintaining the Na+:Ca2+ ratio less than 25:1 for high molecular weight Alg and 3:1 for low molecular weight Alg [11, 22]. The slower degradation of Alg-beads coated with thin polymer films could be due to the delayed reverse diffusion of free Ca2+ from the interior of the particles to the incubating solution. Immersion of Ca2+-saturated alginate beads into a solution that does not contain Ca2+ creates a big difference in the osmotic equilibrium, which causes the entrapped Ca2+ in the beads matrix to diffuse in the solution. GO has a high density of negative surface charges and probably slows down or interrupts the Ca2+ diffusion. Besides, the addition of GO is shown to increase the stiffness of the coatings. These might be the reasons for the significantly increased lifetime of the particles coated with both cross-linked natural polyelectrolytes and synthetic polyelectrolytes.

**Conclusion**

Novel opiorphin loaded alginate particles coated with polyelectrolyte and hybrid polyelectrolyte-GO coatings were constructed. All drug-loaded particles coated with thin polymer films show extended drug-release compared to the uncoated particles, the opiorphin release kinetics was found to depend strongly on the type of the polyelectrolyte coating.
The thin coatings based on strong synthetic polyelectrolytes or on weak natural cross-linked polyelectrolytes are much less prone to degradation and are much better in protecting the Alg-particles from burst drug release compared to the natural non-crosslinked coatings.

Deposition of GO on the top of the polyelectrolyte coatings enhances the stability of the particles, slows down the rate of opiorphin release and thus prolongs the pharmacological activity of the Oph-loaded particles by hours. This effect is more pronounced for the strong synthetic compared to the weak natural polyelectrolyte coatings.

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