PRE-FORMULATION DESIGN OF SUSTAINED-RELEASE GnRHa-LOADED PLGA MICROSPHERES AND ASSOCIATED FORMULATIONS FOR CONTROLLING REPRODUCTION IN AQUACULTURE

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Abstract: Poly(lactide-co-glycolide) PLGA microparticles represent an efficient and modern tool to encapsulate peptide drugs, which enables their administration to live organisms with the benefit of prolonged-release. One area that could make the best of this opportunity is fish breeding in aquaculture. The presented study was centered on the formulation of gonadotropin-releasing hormone (GnRH) analog loaded PLGA microparticles intended for fish breeding augmentation, using the double emulsion evaporation method. In this initial experiment, the influence of several input variables (drug, PLGA type, emulsifier concentration, gelatine concentration in internal phase) on observed parameters (morphology, particle size, drug content, drug release, resuspension index) was evaluated. It was found that at lower emulsifier concentration, the particle size is apparently lower (8.54 ± 6.13-10.36 ± 4.65 vs. 25.56 ± 18.86), which is more advantageous in injection administration. Encapsulation efficiency ranged from 39.13 ± 8.85 to 75.30 ± 8.83, favoring lower emulsifier concentration, while resuspension index (70.99 ± 6.47%) suggested the possibility of longer-term administration. Dissolution tests revealed prolonged release for ten days, with most of the drug released in 72-96 hrs. A follow-up study discerning polymer type/gelatine concentration was suggested. Accompanying studies of imaging agent coumarin-6 encapsulation for eventual distribution imaging and metoclopramide base drug release for potential adjuvant use were also successfully realized.

Keywords: GnRH analogs, microparticles, solvent evaporation, gelatine, sustained drug release, fish reproduction

One of the existing approaches to increase the yield of the reproductive process in fish farming is hormone therapy, used to induce both ovulation and spermatiation (1). For this purpose, gonadotropin-releasing hormone (GnRH) and its analogs (GnRHa), mainly in the form of agonists, can be administered. Numerous studies reporting their positive effect on various species have been published (2, 3). Moreover, dopaminergic agents prevent neuropeptides’ synthesis or block their release from the pituitary gland in fish species with dopamine inhibition (4, 5). Therefore, GnRH or its analogs can be combined with dopaminergic inhibitors like metoclopramide or domperidone to improve the therapy effect further (1).

However, this method of use also brings challenges based on pharmacological/pharmaco-technical aspects. If administered orally, GnRH analogs are eliminated by harsh conditions in the gastrointestinal tract (GIT). If administered parenterally as simple suspension, unstable water-soluble GnRH analogs remain unprotected against enzymes, and their half-life is relatively short (6). It can result in the need for repeated administration. However, it

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is well-known that recurring handling of the treated population causes stress, and it can negatively affect the quantity and quality of gametes (7). Therefore, it is logical to explore possibilities for encapsulating such agents. The solution can be seen in polymeric microparticle carriers. With an appropriate polymer as a carrier, microparticle dosage forms can offer protection against the environment, decrease the single-dose amount, systemic side effects, reduce administration frequency, and the possibility of dose dumping, resulting in so-called controlled drug delivery. It effectively means that after an administration of such a dosage form, the drug is delivered in therapeutic dose for a prolonged time interval without the need for re-administration (8). This concept could also be advantageous in fish, especially in breeding populations (9).

Several polymers were examined as microparticle carriers in fish, including chitosan (10) or copolymer of ethylene and vinyl acetate (11). Very promising are polyesters, such as poly(lactide-co-glycolide) (PLGA) and poly(L-lactide) (PLLA), previously successfully used as antigen carriers in fish (12, 13). They can release an active agent over a more extended period, and due to their biodegradability and biocompatibility, they are very well tolerated systems (14), FDA approved for human use. Therefore, it is clear they make a strong candidate for use in fish reproduction as a GnRHa carrier (15), especially in cases when a combination of PLGA with some synthetic GnRH analogs has not been tested yet.

Before a final formulation design is devised, the pre-formulation study is usually performed to estimate the preparation system behavior and set the basic parameters. Therefore, the main objective of the presented paper is the formulation preparation of GnRHa-loaded PLGA microparticles and their subsequent evaluation. To our knowledge, the selected combination of PLGA and alarelin as GnRHa was not previously studied in fish. Emphasis was placed on prolonged drug release achievement and verification of the microparticles’ applicability to a living organism concerning the pharmaceutical-technological parameters to build a foundation for further in vivo testing. Because the chosen preparation method is essentially simple and with certain analytical backgrounds quite feasible outside the pharmaceutical technology laboratory, an explanation is given at individual evaluations to clarify their practical impact on dosage form administration. Last but not least, a simple metoclopramide base dissolution study was included to complete the outline of possible adjuvant treatment, and the design of fluorescent microparticles was suggested for eventual distribution and immunology studies.

**MATERIAL AND METHODS**

**Materials**

The main drugs used for the study were GnRH analogs alarelin acetate and leuprorelin acetate (both APEX BIO, USA). Coumarin-6 (Sigma Aldrich, USA) served as a model substance for fluorescence study. Gelatine (Sigma Aldrich, USA) was used as an inner aqueous phase thickener to increase a microparticle core density. As a polymer carrier poly(lactide-co-glycolide) acid (PLGA) polymers were used: Resomer® RG 504S (50:50 lactide:glycolide ratio), Resomer® RG 653H (65:35 ratio) and Resomer® RG 753S (75:25 ratio; all Evonik, Germany). Dichloromethane (Penta, Czech Republic) was the organic solvent used for the oil phase, polyvinyl alcohol (PVA; Mw 31,000–50,000; 98–99% hydrolyzed) (Sigma Aldrich, USA) acted as an emulsifier, and sodium chloride (Dr. Kulich Pharma, Czech Republic) as an inorganic salt and osmolyte. Ball-milled (PM 100, Retsch, Germany) metoclopramide (Carbosynth, United Kingdom) intended as an adjuvant was used in the form of standard suspension, whose drug release was also evaluated. Phosphate buffer solution pH 7.0 for in vitro release consisted of 5 g of potassium dihydrogen phosphate and 11 g of potassium hydrogen phosphate (both Dr. Kulich Pharma, Czech Republic) in 900 mL of purified water. All materials were of Ph. Eur. quality.

**Microparticles preparation**

Alarelin and leuprorelin-loaded microparticles were obtained by the double emulsion (water/oil/water, w/o/w) solvent evaporation method. Precisely 800 mg of polymer were dissolved in 5 mL of dichloromethane, representing the oil phase (o). For the inner aqueous phase (w) formation, 10 mg of alarelin or leuprorelin were weighed and dissolved in 1.5 mL of gelatine solution (2.5 or 9.09%). The drug-gelatine solution was added to the oil phase. This mixture was pre-mixed by vortex (Lab Dancer, IKA-Werke, Germany) for 30 seconds. Then the mixture was stirred by homogenizer (T25 basic, IKA-Werke, Germany) at 11 000 rpm for 60 seconds to form a simple w/o emulsion. The drug-gelatine solution was dispersed in the polymer solution. Gelatine solution acted not only as the drug solvent but also as a thickener, increasing the inner water phase viscosity (16). If simple water is used instead of gelatine, the stability of the primary emulsion
would be decreased, and the encapsulation efficiency would be an order of magnitude lower (17).

Consequently, 12 mL of 1% PVA solution were added to the w/o emulsion (besides the A653-G9,1 sample, where 0.1% PVA was used for pre-mixing), and the mixture was stirred by homogenizer at 11 000 rpm for 60 seconds. PVA serves as an emulsifier, which improves the dispersion of the emulsion phases. Finally, after the pre-mixing step, the concentrated double emulsion was poured into a 200 mL solution consisting of 0.1% PVA/2% NaCl solution, basically resulting in diluted w/o/w emulsion. NaCl is osmotically active, slowing the leakage of the hydrophilic drug from the internal w phase. The organic solvent was then evaporated under the mechanical stirrer (Heidolph RZR 2021, Sigma Aldrich, USA) at 450 rpm for 120 minutes, and thus the droplets slowly solidified into spherical particles. The resulting micro-suspension was passed through a 250 μm mesh sieve to remove agglomerates, and microparticles were collected by centrifugation (EBA 20, Hettich, Germany). The obtained particles were resuspended, frozen, and lyophilized (L4-55 PRO, Gregor Instruments, Czech Republic). The lyophilization was carried out until probe and sample temperature evened out and then for another eight hours to ensure secondary drying.

The list of prepared samples with their formulation parameters is summarized in Table 1. The first letter in the sample nomenclature denotes drug (A - alarelin, L - leuprorelin, C - coumarin-6), followed by a number specifying PLGA type (504, 653, 753), ended with G for gelatine (if used) and its concentration in % (2.5, 9.09). Eventually, the last number in the lower index denotes PVA concentration for pre-mixing. If not present, 1% PVA was used. Sample containing coumarin-6 was prepared similarly but using simple w/o solvent evaporation method. The samples A504-G2.5 and A653-G9 consisted of three batches, four runs each, whereas the samples A653-G9,1 used 0.1% PVA for the pre-emulsion step; L653-G9, 753-G9, and C753 consisted of one batch only from 4 runs. A blank sample (753-G9) was prepared under non-pyrogenic conditions (depyrogenation in acetone (Penta, Czech Republic)), followed by heating at 250°C for 90 minutes, using water for injection and laminar box for preparation.

### Microparticles evaluation

#### Drug content analysis

The alarelin and leuprorelin content in PLGA microparticles was determined by high-performance liquid chromatography (HPLC). Firstly, the PLGA microparticles were dissolved in acetone, and the resulting solution was mixed with phosphate buffer of pH 7.0 in a 1:1 ratio (v/v). This mixture was filtered through a 0.45 μm membrane filter. The content was then quantified by the HPLC (Agilent 1100, Agilent Technologies, USA) involving NUCLEODUR 100-5 CN-RP (150 mm × 4.6 mm, 5 μm) column. A binary mixture of acetonitrile: 20 mM H3PO4 (16:84, v/v) was used as a mobile phase at 0.8 mL/min flow rate with a temperature set at 30°C, injected sample volume of 20 μL and detection wavelength of 220 nm.

The coumarin-6 content from sample C753 was determined by a UV/Vis spectrometer (Lambda 25, Perkin Elmer, USA) at 450 nm. The samples were prepared by dissolving 100 mg of microparticles in 10 mL of dichloromethane and filtered through a 0.22 μm membrane filter. This procedure was conducted in triplicate.

Mean values and standard deviations (SD) were expressed from the obtained values. Encapsulation efficiency (%; EE) [1], drug load (%; DL) [2], and practical yield (%) [3] were also determined by using the equations below (18, 19, 20).

\[
EE = \frac{W_1}{C_t} \times 100 \% \quad [1]
\]

### Table 1. The microparticles samples and their formulation/process characteristics

| Sample* | PLGA polymer** | Drug, amount | Gelatine solution - volume (mL), concentration (%) | PVA (%) | Method      |
|----------|----------------|--------------|--------------------------------------------------|---------|-------------|
| 753-G9   | 753S           | -            | 1.5, 9.09                                        | 1.0     | w/o/w      |
| A504-G2.5| 504S           | Alarelin, 10 mg | 1.5, 2.5                                        | 1.0     | w/o/w      |
| A653-G9  | 653H           | Alarelin, 10 mg | 1.5, 9.09                                        | 1.0     | w/o/w      |
| A653-G9,1| 653H           | Alarelin, 10 mg | 1.5, 9.09                                        | 0.1     | w/o/w      |
| L653-G9  | 653H           | Leuprorelin, 10 mg | 1.5, 9.09                                        | 1.0     | w/o/w      |
| C753     | 753S           | Coumarin-6, 500 µg | -                                                 | 1.0     | o/w        |

*A - alarelin; L - leuprorelin, G - gelatine; **the first two numbers express the ratio of lactic acid monomers in PLGA, the third number represents the PLGA viscosity (dL/g), S - ester terminated, H - free carboxylic acid
\[ DL = \frac{w_1}{w_2} \times 100 \% \]  \hspace{1cm} [2]

\[ Yield = \frac{w_2}{w_t} \times 100 \% \]  \hspace{1cm} [3]

where: \( w_1 \) represents the actual weight of the drug in microparticles; \( c_t \) is the theoretical amount of drug; \( w_2 \) is the total weight of prepared microparticles; \( w_t \) is the theoretical yield (total amount of drug and polymer used for the microparticle preparation).

**Scanning electron microscopy (SEM)**

The morphology and properties of samples were evaluated using scanning electron microscopy (MIRA3, Tescan Orsay Holding, Czech Republic) equipped with a secondary electron detector. Using carbon conductive double-sided adhesive tape, a sample was mounted on an SEM specimen stub (Agar Scientific, United Kingdom). Microparticles were coated by a layer of gold (20 nm) to eliminate charging artifacts using the metal sputtering coating method with argon atmosphere (Q150R ES Rotary-Pumped Sputter Coater/Carbon Coater, Quorum Technologies, United Kingdom). SEM images were obtained at an accelerating voltage of 3 kV.

**Optical microscope analysis**

Morphological properties of the prepared microparticles (sphericity factor, equivalent diameter) were evaluated using an optical microscope (Nikon Eclipse E200, Nikon, Japan) coupled with a camera (72AUC02 USB, The Imaging Source, Germany). Randomly selected 200 microparticles were evaluated by computer software (NIS-Elements AR 4.0, Nikon, Japan). An image of every sample was also taken.

Sphericity factor (SF) is a dimensionless unit describing particles shape. Equivalent diameter (ED) determines the circle diameter having the same area as the object observed. Both parameters can be determined according to the equation below:

\[ SF = \frac{4\pi A}{p^2} \]  \hspace{1cm} [4]

\[ ED = \frac{4A}{\pi} [\text{mm}] \]  \hspace{1cm} [5]

where: \( A \) represents area in mm\(^2\) and \( p \) is microparticles perimeter in mm (21).

**Laser diffraction**

Laser diffraction was performed using the laser particle size analyzer to determine size distribution (LA-960, Horiba Scientific, Japan). The real refractive index was set to 1.6. The imaginary refractive index was neglected. Sufficient sample weight was resuspended in 1 mL of purified water to induce signal. Resuspended samples were placed into the device, measured immediately, and analyzed for volume-based size distribution. The measurements were performed in triplicate. The results were expressed as median, mean values with SD, and the diameters at the 10th, 50th, and 90th percentiles of the cumulative undersize plot (D10, D50, D90).

**Confocal microscope analysis**

The water suspension of the C753 sample (10 mg/mL) was placed into µ-Slide 8 Well (Ibidi GmbH, Gräfelfing, Germany). After sedimentation, microparticles were observed using a confocal microscope (Leica SP8, Leica, Germany). A 458 nm wavelength laser was used for the excitation, and a hybrid emission detector (HyD) was set to 530-560 nm. The confocal microscopy images were acquired with a Leica objective HCX PL APO 63x.

**In vitro release studies**

**In vitro** alarelin/leuprorelin release in buffer solution: **In vitro** release testing of alarelin (time course ten days) was performed in Franz cells connected in series at 5 ± 0.5°C without stirring. Exactly 20 mg of microparticles were placed into a cell containing 20 mL of phosphate pH 7.0 buffer solution. The release medium was sampled (1.0 mL) regularly every 24 hours, followed by a complete replacement of the dissolution medium in cells. All measurements were performed with six units. Alarelin concentration was quantified by HPLC using the method stated in the Drug content analysis section. The dissolution test of leuprorelin was conducted in the same fashion except that 50 mg of sample were weighed into 30 mL of pH 7.0 buffer (22). The pH value varies in different tissues and fish species (23). However, the flesh pH is very often close to pH 7.0 (24). Therefore it was chosen as a reasonable compromise.

**In vitro** alarelin release in agar: The method was based on the adjusted procedure used in the release study of GnRHα-loaded polyanhydride microparticles (25). The selected samples A504-G2.5 and A653-G9 were tested in agar gel under three conditions. Exactly 50 mg of microparticles were suspended in 1%/0.4 mL, 1%/0.8 mL, or 2%/0.4 mL agarose solution in a glass vial, cooled down to let the agarose solidify, and then another
Resuspension index

Resuspension index (RI) expresses the percentage of resuspended microparticles remaining resuspended in a medium after a given time. Exactly 30 mg of microparticles were placed in a 1.5 mL Eppendorf tube. 1 mL of distilled water was added, and it was vortexed for 1 minute. This suspension was left to stand for 10 minutes to allow the aggregated particles to sediment. The formed suspension was gently collected into a centrifuge tube. This procedure was performed in a total of three consequent repetitions. The collected suspension was centrifuged at 1500 rpm for 10 minutes, and the supernatants were removed. Resuspended and aggregated fractions were lyophilized and weighted. Triplicate determinations were performed for the 753-G9 sample, and the result RI was calculated as:

\[
RI = \frac{m_r}{m_r + m_a} \times 100 \% \tag{6}
\]

where: \(m_r\) expresses the resuspended mass and \(m_a\) the aggregated mass.

**Pierce chromogenic endotoxin test**

Each sample in 200 mg was dispersed in 2 mL of endotoxin-free water. The supernatant was collected from the settled suspensions, and endotoxin levels were tested using the Pierce test (Pierce Chromogenic Endotoxin Quant Kit, Thermofisher Scientific, USA).

**RESULTS AND DISCUSSION**

Alarelin and leuporelin-loaded microparticles were successfully formulated by the double emulsion \((w/o/w)_2\) method using the pre-mixing step. The creation of the concentrated double emulsion prevented droplets’ coalescence during the process. It resulted in final microparticles with particle size in tens of microns, considered optimal for non-problematic injectability (28). Based on data available in scientific literature, the PLGA microparticles prepared without this step exhibited significantly bigger particle sizes in hundreds of microns (29). Coumarin-6-loaded particles were also successfully prepared by the o/w method.

**Encapsulation process and yield**

Table 2 shows the yield and drug content analysis results. These parameters give information about the efficiency and loss rate of the preparation process. In this case, it provided a satisfactory yield, as the lowest value was 73.97% for L653-G9. The highest was found in Resomer® 653H sample A653-G9, (83.14 ± 2.54%). In 1% PVA alarelin samples, the EE and DL analysis turned out to be in favor of Resomer® 504 with EE of 46.56 ± 6.18% and DL 0.55 ± 0.07%. The differences from particles prepared with Resomers® 653H can be attributed to the higher viscosity of the Resomer® 504 (30). Higher polymer viscosity produces lower yield through process losses (adherence to equipment). On the other hand, higher viscosity increases drug entrapment, resulting in higher values of EE and DL (31). Nevertheless, since the values overlap when SD is considered, the EE and DL of both samples could be seen as comparable, and further investigation will be required. Comparing A653-G9, and A653-G9.01 samples clearly shows that the lower PVA concentration used for the pre-mixing step resulted in significantly higher EE and DL. This observation is very probably related to their higher mean size. The overall surface is lower, and the drug thus has less opportunity to leak during the preparation process (32). However, a higher mean size may cause problematic administration via syringe; therefore, a final formulation may be understood as a compromise.
The leuprorelin sample L653-G9 showed a favorable result with EE of 58.98 ± 1.77%, compared to the corresponding alarelin sample. Excellent EE was reached in the sample C753 with the value of 91.50 ± 0.96%, which resulted from the coumarin-6 lipophilic character (33).

Morphology and particle size

SEM analysis (Figure 1) showed excellently spherical particles for each prepared sample. It revealed a smooth surface without cracks, and it hinted at lower porosity with decreasing glycolic acid content in a polymer (34). Resomer® 504S (Figure 1 - C, D) provided particles with numerous pores; meanwhile, Resomer® 753 (Figure 1 - A, B) yielded particles without visible pores. The images also suggested partially hollow particles, mainly in the Resomer® 504S sample. By estimation, particle size ranged in the interval 1-30 µm. It means polydisperse particles with relatively heterogeneous size distribution with included agglomerates. Figure 1 - G, H also indicates bigger particles in the sample A653-G9, pre-mixed with 0.1% PVA than in the corresponding 1% PVA sample, confirming the

| Sample     | EE (%)         | DL (%)    | Yield (%) | Mean size (µm) | Sphericity factor |
|------------|----------------|-----------|-----------|----------------|------------------|
| 753-G9     | -              | -         | 75.36     | 6.25 ± 2.51    | 0.999 ± 0.001    |
| A504-G2.5  | 46.56 ± 6.18   | 0.55 ± 0.07| 77.01 ± 0.78| 8.54 ± 6.13    | 0.973 ± 0.027    |
| A653-G9 1/  | 39.13 ± 8.85   | 0.41 ± 0.09| 83.14 ± 2.54| 10.36 ± 4.65   | 0.999 ± 0.001    |
| A653-G9 0.1| 75.30 ± 8.83   | 0.80 ± 0.09| 75.32     | 25.56 ± 18.86  | 0.999 ± 0.001    |
| L653-G9    | 58.98 ± 1.77   | 0.62 ± 0.02| 73.97     | 8.72 ± 4.31    | 0.999 ± 0.002    |
| C753       | 91.50 ± 0.96   | 0.06 ± 0.00| 74.30     | 7.19 ± 2.80    | 0.998 ± 0.003    |

EE = encapsulation efficiency; DL = drug loading

Figure 1. SEM analysis – A) 753-G9 sample (bar = 10 µm); B) 753-G9 sample (bar = 50 µm); C) A504-G2.5 sample (bar = 10 µm); D) A504-G2.5 sample (bar = 50 µm); E) A653-G9, sample (bar = 10 µm); F) A653-G9, sample (bar = 50 µm); G) A653-G9, sample (bar = 10 µm); H) A653-G9, sample (bar = 50 µm); I) L653-G9 sample (bar = 10 µm); J) L653-G9 sample (bar = 50 µm)
fundamental influence of PVA concentration on particle size (35).

Optical microscope analysis followed, allowing particle size and sphericity measurement (Table 2). Images of the prepared microparticles are shown in Figure 2. In general, every prepared sample consisted of highly spherical particles as the sphericity factor ranged between 0.973 ± 0.027 and 0.999 ± 0.001. For comparison, a pellet sphericity factor of 0.9 is usually stated as sufficient (36). This feature is highly desirable because high sphericity means better flow properties of the dry powder. It also gives a large surface for eventual resuspension in the injection medium. The particle size prepared using 1% PVA took values from 6.25 ± 2.51 to 10.36 ± 4.65 µm, suggesting a parenterally applicable dosage form into fish organisms (37, 38). The sample A653-G90.1 pre-mixed with 0.1% PVA exhibited a two times higher particle size (25.56 ± 18.86 µm), confirming observations and conclusions reached in previous paragraphs. In addition to the optical microscope analysis, for the C753 sample, the confocal microscope analysis was performed to prove the sample functionality. Figure 3 shows that the particle manifested detectable fluorescence. This feature can be used for tissue deposition studies in vivo to confirm correct administration or in immunology studies.

Finally, laser diffraction was performed to complete the particle size examination (Figure 4 and 5, Table 3). The median was approximately 20 µm for all 1% PVA samples. Diameter values on cumulative % were ranged from 7.8 to 13.2 µm (10%), 15.4 µm to 26.2 µm (50%) and 41.4 µm to 64.2 µm (90%). The laser diffraction also confirmed previous observations that the sample A653-G90.1 with a median of 35.7 µm provides a significantly higher particle size. Wide distribution interval (Figure 4, 5) and higher SD indicate polydisperse samples. Compared to the optical microscope analysis, the particle size

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**Figure 2. Optical microscope analysis – A) 753-G9 sample (bar = 50 µm); B) A504-G2.5 sample (bar = 50 µm); C) A653-G9, sample (bar = 50 µm); D) A653-G90.1 sample (bar = 50 µm); E) L653-G9 sample (bar = 50 µm); F) C753 sample (bar = 50 µm).**

**Figure 3. Confocal microscopy analysis of sample C753: A) bar = 56.65 µm; B) bar = 14.62 µm; C) 3D projection.**
measured by laser diffraction is higher. Since microscope images clearly show particle size, the diffraction results suggest the presence of microparticle agglomerates, previously visible in SEM analysis results. They could be caused by imperfect resuspension in water. A solution could be seen in using a surfactant for better wetting (39, 40). Therefore, a medium for eventual in vivo administration should contain a surfactant to facilitate injectability. A surfactant should be selected based on its properties, interaction potential, and toxicological profile to minimize potential adverse effects (41).

**In vitro release studies**

**GnRH analogs and metoclopramide dissolution test**

A dissolution test is a form of evaluation, which, among other things, helps estimate behavior in terms of drug release. Figure 6 shows that almost the whole alarelin amount was released during the first hours of the Franz cells dissolution test. The sample A653-G9 released a significantly smaller drug amount than the A504-G2.5 sample. It could be attributed mainly to the 9.09% gelatine solution used as an inner aqueous phase thickener. When
solidified, it is a very rigid thermoreversible gel. Only the outer layer of a gelatine microparticle core was hydrated after contact with an aqueous medium. It could be a reason for such limited drug release compared to the 2.5% gel used in the corresponding sample. This finding can be supported by experimental data published by Yu et al. In this study, the 5% gelatine gel of the inner water phase ensured a sustained release of highly soluble losartan potassium. Although the molecular weight of this drug is significantly lower (Mw 461.0) than that of alarelin (Mw 1167.3), a prolonged-release for 30 days has been observed (42). Figure 6 also shows that leuprorelin lost its stability over time and was decomposed in an aqueous medium. Thus, this sample was discarded for subsequent dissolution tests.

Due to the drug’s immediate release during the dissolution test in buffer solutions, it was decided that a model imitating parenteral tissue administration is needed to obtain a more accurate preview. The previously suggested gel dissolution (25) was optimized and adjusted for the current experiment. Figure 7 shows that the drug dissolution in gels was considerably slower with effective drug release at least in the interval of 48-72 hrs. The difference between samples in terms of gelatine concentration has been preserved, as A504-G2.5 released twice the amount of A653-G9. The various adjustments to gel layers did not significantly influence the drug release. The only mild difference can be seen in sample A504-G2.5 in 0.8 mL/1% agarose gel, as this dissolution profile is slightly slower than its counterparts. It is for further investigation to decide the effect of polymer selection and gelatine concentration on drug release. However, the results showed potential for drug release profile modulation, which could be helpful when treating different fish species with varying reproduction requirements. Not to mention
the more samples can be easily combined in one dose.

The dissolution behavior of the metoclopramide suspension was investigated using a non-compendial exploratory biphasic dissolution method. It is a possible approach to assess the concurrent in vivo drug absorption process, and the used organic phase (octanol) simulates the transition through lipophilic cell membranes (43). The dissolution profile of the metoclopramide suspension (Figure 6) indicates that the drug gradually merged into an organic phase within ten days. Metoclopramide is a dopamine receptor antagonist, which can be used as an adjuvant during the GnRHa treatment. It increases therapy efficacy by blocking endogenous dopamine inhibition of the GnRH axis. In combination with the alarelin-loaded microparticles, this dissolution profile could be suitable for improving the reproduction of commercially farmed fish species. The obtained profile also gives a better insight into its behavior in tissues with more liquid content, like peritoneum, which can be beneficial when deciding the dose.

**Resuspension index**

Resuspension index (RI) was determined to assess the reproducibility of a drug dosing during a longer time interval when a microparticle suspension from one batch/package is used to treat multiple humans or animals at the same time. The higher RI corresponds to the slower particle sedimentation and the drug’s exact dosing without repeated resuspending. The RI can be evaluated at different time intervals (44, 45), depending on the internal requirements and expected duration of specimen administration. The presented study assessed the RI for the drug-free 753-G9 microparticles exhibiting a comparable particle size to the alarelin-loaded sample. The obtained value was 70.99 ± 6.47%, meaning most of the particles remained resuspended, and the suspension is usable. Nevertheless, brief vortexing in regular intervals would be desirable for reproducible doses during fish population inoculation.

**Pierce chromogenic endotoxin test**

Figure 8 shows that all tested samples were contaminated by endotoxins to various degrees (0.29–1.66 IU/mL). Pierce chromogenic endotoxin test revealed that the o/w method is less susceptible to lipopolysaccharide fragments (LPS) contamination than the w1/o/w2 technique. It points to gelatine as the most critical factor (46). Despite using gelatine suitable for microbiology and depyrogenation protocols, it wasn’t possible to prepare fully uncontaminated microparticles using any method. Thus, outside the preparation of experimental dosage form, it would be necessary to establish microparticles preparation under the cleanroom conditions to prevent LPS contamination. Also, unique methods of adjusting gelatine to decrease LPS content are available (47). However, it should be noted that unlike higher animals, which are extremely sensitive to endotoxins even at low doses, lower vertebrates like fish are often resistant to endotoxic shock (48).

**CONCLUSION**

A comprehensive approach to controlling aquaculture reproduction was suggested during the pre-formulation study. The PLGA microparticles prepared by the w1/o/w2 evaporation method were shown to be a potentially suitable carrier for the long-term release of GnRH analog alarelin. Their combination with metoclopramide suspension releasing the drug in a prolonged manner was demonstrated as a viable option. As a part of a microparticle core, the gelatine was revealed to be a key factor modifying in vitro drug release characteristics, as was demonstrated using dissolution test in agar medium mimicking in vivo conditions. A follow-up study was suggested to investigate polymer type/gelation concentration. Excellent characteristics, including high yield,
regular spherical shape, suitable particle size, and resuspension behavior, open the possibility of their trouble-free application by injection into a fish body. LPS contamination, originating probably from using gelatine solution, drew attention to the problem that would have to be solved in the case of broader use.

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Conflicts of interest

The authors declare no conflict of interest.

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