# Quantification of biodegradable PLGA nanoparticles for drug targeting

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**Objective.** The aim of this work was the development of appropriate analytical methods and assays for determining and monitoring composition and degradation of nanoparticles built from PLGA (poly D, L-lactid-co-glycolid), which can be reloaded with different drugs. A sensitive and precise method for monitoring of nanoparticle degradation in vitro was developed and optimized. Nanoparticles allow a selective enrichment of different drugs and knowledge of the nature and type of their degradation is essential for characterization and control of drug release and dosage. **Materials and methods.** The first method developed during this work to quantify the PLGA polymer matrix use advantage of the chemical reaction of aliphatic carboxylic acids with ferric chloride ($FeCl_3$) thus quantifying both degradation products of PLGA, lactic and glycol acids, at the same time. A second assay method of choice was to react to the polymer hydrolysate with lactate dehydrogenase, thus assaying selectively the lactic acid part. **Results.** During development of both of described methods was possible to determine dynamic range for PLGA matrix and nanoparticles, as well as to characterize impact of Pluronic F-68 and glycolic acid on lactate dehydrogenase activity. **Conclusion.** During our work we were able to develop two sensitive methods for monitoring of biodegradation of polymers which are consecutively used as a nanoparticle matrix in drug targeting.

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**Key words:** Drug targeting, Nanoparticles, Poly (lactid-co-glycolic) acid, Biodegradation.

## Introduction

Recent developments in the field of medical nanotechnology and the possibility of chemical engineering of particles smaller in size than human cells allowed biochemical and pharmaceutical technology to realize drug targeting, which was already a vision since several decades. One possibility to gain increased enrichment of drugs in the respective tissue which has to be treated selectively is the reversible binding of drugs to nanostructures biocompatible carrier systems.

Such carriers are in the size of only a few nanometers thus being 100 to 200 fold

smaller than human cells. Examples for such carrier systems are spherical liposome and nanoparticles consisting of degradable, biocompatible polymers. Their small size combined with chemical engineering of their surface to bind selectively to the respective cells of choice, allow a directed enrichment of the drugs occluded in the degradable particles in diseases tissues and organs. Nanoparticles consisting of a (bio) degradable polymer matrix may carry the respective drugs either occluded or reversibly adsorbed. Various polymers have been used in drug delivery research that can effectively deliver the drug to a target site and increase the therapeutic effect, while minimizing any other side effect (1, 2).

One of the most important advantages of nanoparticles is their ability to cross physiological epithelial barriers because of their variable diameter in range from 10 nm to 1000 nm.

Colloidal carriers such as nanoparticles based on poly-lactic-co-glycolic acid (PLGA) have been extensively studied in medical and biotechnological sciences as drug delivery systems, which were suitable for most administration routes.

Poly (D, L-lactide-co-glycolide) (PLGA) was approved in the year 2000 by FDA (Food and Drug Administration) for application in drug targeting (3, 4).

Another unique properties of PLGA such as biocompatibility, biodegradability and controlled release, make this polyester one of the most promising material in medical and biotechnological research with high commercial interest.

It's also shown that PLGA has bioadhesive properties and binds to mucosa of gastrointestinal tract (5, 6, 7).

This effect can increase the residency time and may enhance the drug absorption time due to intimate contact with the epithelium cells. Also, biodegradable nanoparticles are of particular interest as they provide protection of fragile molecules against enzymatic and hydrolytic degradation in the gastrointestinal tracts.

In medicine PLGA is well known as a restorable suture material in surgery, which is randomly hydrolyzed in the organism to their biocompatible metabolites, lactic and glycolic acid.

Lactic acid is converted to pyruvate, which is degraded in the Krebs cycle via acetylating of coenzyme A, and carbon dioxide, which is mainly eliminated by respiration. Part of the glycolate is excreted directly via the urine; another part is oxidized to glyoxylate, which is converted to glycine, serine and pyruvate. (8, 9)

The rate of biodegradation depends on the lactic/glycolic acid ratio a 50/50 PLGA, as was used in this work, is degraded fully within one month.

The nanoparticles used were synthesized by double emulsifying techniques showing the advantage that hydrophobic as well as hydrophilic drug as e.g. therapeutic relevant peptides and proteins may be entrapped.

Up to now it quantification of nanoparticles from biodegradable and biocompatible PLGA was done by gravimetric methods after lyophilisation. Unfortunately this method allows only for quantification of the pure polymer, whereas in complex systems as in the aforementioned drug containing nanoparticles also the occluded drug and additives from the synthesis contribute gravimetrically and impair a correct result.

The object of the methods developed during this work to quantify the polymer matrix was to primarily hydrolyze the nanoparticles quantitatively and assay the monomers. One analytical technique was to take advantage of the chemical reaction of aliphatic carboxylic acids with ferric chloride ($FeCl_3$) thus quantifying both degradation products at the same time.

A second assay method of choice was to react to the polymer hydrolysate with lactate

dehydrogenase, thus assaying selectively the lactic acid part.

## Materials and methods

*Materials*

Resomer 503 H (Poly (D, L-lactide-co-glycolic acid) (PLGA, lactide:glycolide = 50:50, inherent viscosity 0.32-0.44 dl/g, acid number > 3 mg KOH/g) was supplied by Boehringer Ingelheim (Ingelheim, Germany).

Glycylglycin hydrochloride, L-Glutamic acid, DL-lactic acid lithium salt, Glycolic acid, D-(-)-Lactic acid, L-(+)-Lactic acid, NAD: β-Nicotinamide adenine dinucleotide hydrate, from Yeast, L-lactic dehydrogenase, from Bovine Muscle, 686 Units/mg protein, D-Lactic Dehydrogenase from Lactobacillus leichmannii, 282 Units/mg protein, Glutamic-Pyruvic Transaminase, from Porcine Heart, 90 Units/mg protein, and Pluronic F-68 were purchased from Sigma- Aldrich (Vienna, Austria).

*Methods*

**Preparation of PLGA nanoparticles**

The nanoparticles were prepared by a water-in-oil-in water solvent evaporation technique which allows for entrapment of hydrophobic as well as hydrophilic drugs as e.g. therapeutic relevant peptides and purified via diafiltration using Vivaflow 50 to remove Pluronic F68, an additive essential for nanoparticle preparation and stabilization.

The amount of PLGA in nanoparticles after preparation procedure was quantified by lyophilisation. The nanoparticles were finally stored at -80°C until use.

**Optical test for quantification of D, L-lactic acid**

The optical enzymatic test for quantification of the amount of lactic acid in PLGA nanoparticles provides a sensitive method for continuous monitoring of lactate-dehydrogenase activity *in vitro* using a photometer. Lactate-dehydrogenase converts or hydrolyzes D, L- lactic acid, in presence of NAD, to pyruvate. In the final step of this enzyme-coupled assay, pyruvate will be converted to L-alanine in the presence of L-glutamate by the action of glutamate- pyruvate transaminase and also removed from reaction.

$$\text{Lactate} + NAD^+ \xrightarrow{\text{Lactate-dehydrogenase}} \text{Pyruvate} + NADH + H^+$$

$$\text{Pyruvate} + \text{L-Glutamate} \xrightarrow{\text{Glutamate-pyruvate transaminase}} \text{L- Alanine} + \text{2-Oxoglutarate}$$

## Reaction with ferric (III) chloride

Chemical reaction of aliphatic carboxylic acids with ferric (III) chloride results in specific color change whose intensity is strongly dependent from pH value of solution.

In a strong acidic solution the color of $FeCl_3$ is pale, whereas in slightly alkaline solution $FeCl_3$ reacts with hydroxide ions to form a colloidal iron (III) hydroxide. Most suitable conditions for reactions with $FeCl_3$ are in solution at pH 7 where the formed complex is stable and can be identified spectrophotometrically by measuring the absorption at 360-370nm or at 450-460 nm (10).

As mentioned above PLGA (poly-lactic-co-glycolic acid) belongs to the family of aliphatic polyesters and contain two aliphatic carboxylic acids, lactic and glycolic acid, in equal amounts. Due to the advantage of the chemical reaction on aliphatic carboxylic acids with ferric chloride ($FeCl_3$), both products of PLGA degradation can be quantified at the same time.

## Results

*Impact of Glycolic acid on the activity of lactate- dehydogenase*

For development of methods to quantify the PLGA polymer matrix it was very important to characterize the nature of interaction

between glycolic acid and lactate dehydrogenase. It's already known from literature that glyoxiclic acid in presence of cofactor NADH and glyoxiclic acid-reductase is converted to glycolic acid (11).

By this reaction a reduction of NADH-absorption 340 nm was measured.

$$\underset{\text{Glyoxiclic acid}}{CHO-COOH} + NADH + H^{+} \leftrightarrow \underset{\text{Glycolic acid}}{HO-CH2-COOH} + NAD^{+}$$

**Experimental procedure**

In order to characterize the type of interaction between glycolic acid and lactate dehydrogenase a stock of 5 mg (0.066 mmol) glycolic acid in 20 ml Glycylglycine buffer was prepared (end concentration of glycolic acid 250 µg/ml).

Standard-dilution of glycolic acid in Glycylglycine buffer in a range of 50 to 250 µg/ml were prepared and incubated in a water bath at 37 °C for 30 minutes.

Enzyme dilutions were diluted with Glycylglycine buffer and their protein amount was measured by Bradford just prior to use.

**Test procedure**

A master-mix containing 5 ml Glycylglycine buffer pH 10, 5 ml distilled water and 1 ml of 47 mM NAD was prepared. This Master-mix was incubated in a water bath at 37°C for 30 min. In each reaction tube, 2 ml of master-mix was mixed with 100 µl of glycolic acid standard dilutions. After incubation for 5 minutes in a water bath at 37°C the absorption of the samples was measured for 1 minute at 340 nm (3 ml SUPRASIL Quartz cuvettes were used). The obtained value was characterized as E1. As a blank we used 2 ml of Glycylglycine Buffer pH 10. Finally, the reaction was started by adding 1.12 U of L-lactate dehydrogenase or D- lactate dehydrogenase to a cuvette with glycolic acid and the absorption was measured every 2.5 minutes over a period of 25 minutes. This obtained value was characterized as E2.

For each measured concentration of glycolic acid a difference $\Delta E = E2 - E1$ was calculated and plotted versus time in [min] and the corresponding slope k was determined.

Finally, the concentration of glycolic acid was applied as a value of x-coordinate versus the corresponding slope k as ordinate values.

Figures 1 and 2 show that glycolic acid acts as a substrate for lactate dehydrogenase under our reaction conditions. But at the same time this molecule acts also as an inhibitor at the active site of this enzyme with respect to the substrate lactic acid.

Table 1 Measured value by reaction of glycolic acid with L-lactate dehydrogenase or D-lactate dehydrogenase

| Concentration of Glycolic acid in µg/ml | Slope *k* after reaction with L-lactate dehydrogenase | Slope *k* after reaction with D-lactate dehydrogenase |
|---|---|---|
| 50 | 0.0011 | 0.0009 |
| 100 | 0.0009 | 0.0012 |
| 150 | 0.0013 | 0.0018 |
| 200 | 0.0011 | 0.0020 |
| 250 | 0.0016 | 0.0013 |

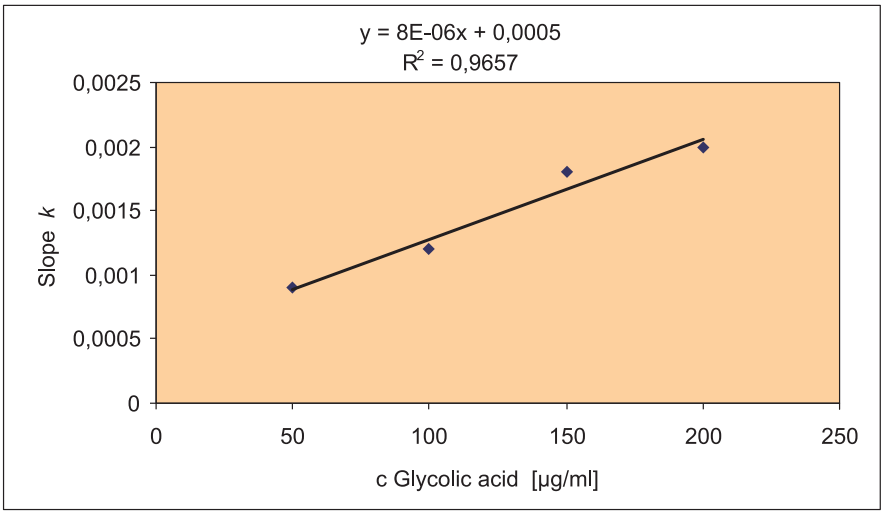


Figure 1 Reaction of Glycolic acid with L-Lactate dehydrogenase by measuring the absorbance at 340 nm

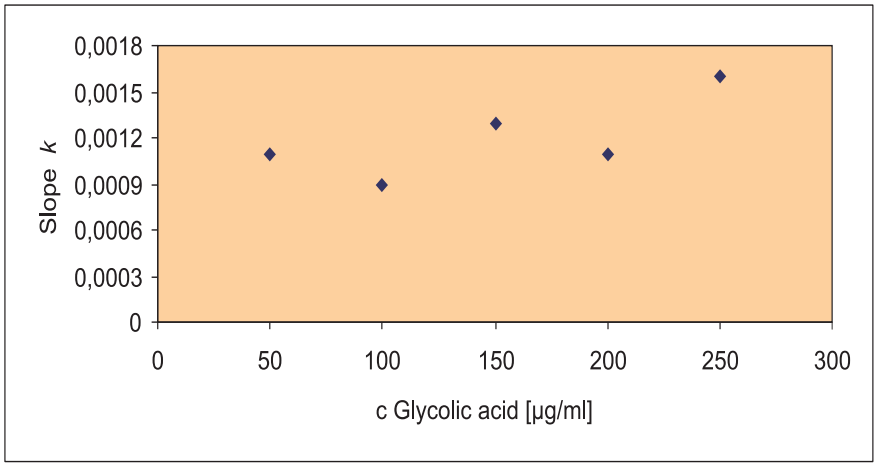


Figure 2 Reaction of Glycolic acid with D-Lactate dehydrogenase by measuring the absorbance at 340 nm

*Determination of critical micelle concentration of anionic surfactant Pluronic F-68*

In pharmacy surfactants are most frequently applied to improve solubility, stabilization and absorption of drug substances. In this work we used the anionic surfactant Pluronic F- 68 as an essential additive for stabilization of nanoparticle suspension in an end concentration of 1. 6% (w/v) (12).

The use of protective surfactants (i.e., polaxamers and poloxamines) could possibly prevent unwanted interactions between the drug and the PLGA as well as neutralize the acidity generated in the course of polymer degradation (13).

In order to characterize an influence of Pluronic F- 68 on the activity of Lactate dehydrogenase it was important to determine a critical micelle concentration of Pluronic F-68 in aqueous solution.

**Experimental procedure**

For determination of the critical micelle concentration of Pluronic F-68 a Sudan III

Table 2 Determination of critical micelle concentration of Pluronic F-68: obtained value

| Concentration of Pluronic F-68 in % (w/v) | $\Delta E = E_{\text{with Pluronic F-68}} - E_{\text{without Pluronic F-68}}$ at 507 nm |
|---|---|
| 0.10 | 0.073 |
| 0.16 | 0.092 |
| 0.25 | 0.093 |
| 0.50 | 0.119 |
| 1.00 | 0.263 |
| 1.50 | 0.389 |
| 2.00 | 0.577 |
| 2.50 | 0.746 |

(Sudan red, $C_{22}H_{16}N_4O$, MW 352.4 g/mol) was used as an indicator.

In 8 different reaction tubes, 2ml of the corresponding Pluronic concentration (0.1%; 0.16%; 0.25%; 0.5%; 1%; 1.5%; 2% and 2.5% (w/v)) was added and mixed with 200 µl $NAD^+$ and 100 µl L-lactic acid in concentration of 100 µg/ml. After 30 minutes of incubation in water bath at 37°C a small amount of Sudan III was added into each reaction vessel. Finally, after additional incubation for 15 minutes at 37°C, all samples were centrifuged (2 minutes by 14 000 rpm) and absorption of the samples was measured at 507 nm. As a blank we used the same preparation without Pluronic F-68.

Figure 3 shows that the absorption of Sudan III increases very abruptly at a concentration of 0.8% as a result of spontaneous micelle formation, and this point was defined as the critical micelle concentration of Pluronic F-68.

### *Optical test for quantification of D, L-lactic acid amount in PLGA and PLGA nanoparticles*

In order to determine the amount of D, L-lactic acid in PLGA and PLGA nanoparticles, an enzyme-coupled assay of lactate dehydrogenase (LDH) was carried out. At pH 10, the activity of the lactate dehydrogenase that oxidizes lactic acid in presence of $NAD^+$ to pyruvate was examined.

#### Generation of a calibration line with L-lactic acid and glycolic acid

Resomer RG 503 H contains D, L-lactic acid and glycolic acid in equal amounts, an ideal ratio of both components for fast deg-

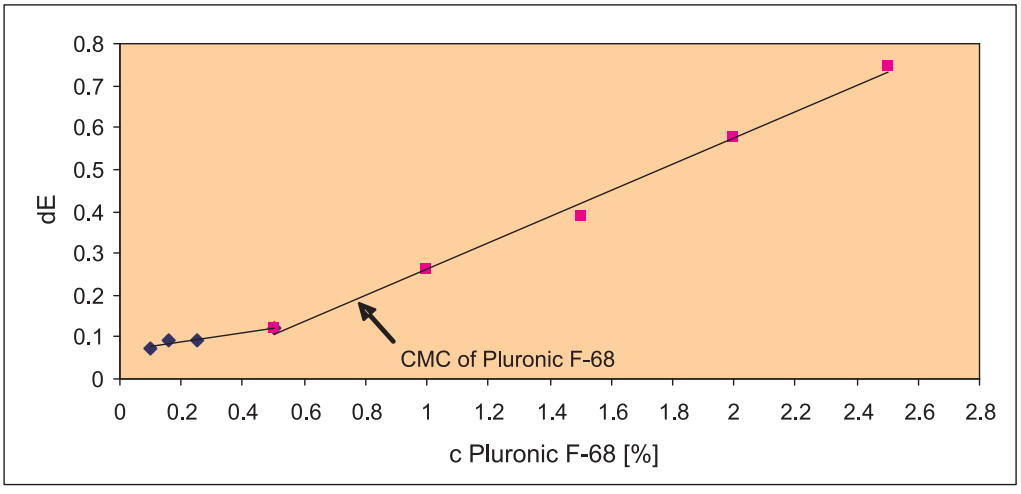


Figure 3 Critical micelle concentration CMC of Pluronic F-68

radation in human body. D, L-lactic acid is chiral and has two optical isomers, L-lactic acid and D-lactic acid. PLGA contains both optical isomers of lactic acid in the same amount. L-lactic acid is the biologically important isomer.

In previous experiment we could show that glycolic acid acts as a substrate and as an inhibitor for lactate dehydrogenase under our reaction conditions.

Therefore, for corrected generation of calibration lines a mixture of L-lactic aid and glycolic acid in a molar ratio 1:2, in the identical ratio as in PLGA was used.

A calibration line was generated by means of increasing L-lactic acid and glycolic acid amount. A stock containing 4 mg (0.044 mmol) L-lactic acid (end concentration 200 µg/ml) and 6.756 mg (0.088 mmol) glycolic acid (end concentration 337.7 µg/ml) in 0.01 M NaOH was prepared. Dilutions of a stock in the range of 50 to 200 µg/ml of L-lactic acid in 0.01 M NaOH were used for generation of a calibration curve. Finally, the pH-value of each sample was adjusted with 1 M HCl to 10 and incubated in a water bath at 37 °C for 30 minutes.

L-lactate dehydrogenase and Glutamate-pyruvate transaminase dilutions were diluted with Glycylglycine buffer pH 10 and their protein amount was measured by Bradford just prior to use.

**Test procedure**

A master-mix containing 7 ml Glycylglycine buffer pH 10, 7 ml distilled water and 1.4 ml of 47 mM NAD was prepared. This master-mix was incubated in awater bath at 37°C for 30 min. In each reaction tube, 2 ml of master-mix was mixed with 100 µl of stock solutions (50 to 200 µg/ml) and 0.126 U of glutamate-pyruvate transaminase. After incubation in water bath for 5 minutes at 37°C

Table 3 Measured value for L-lactic acid-calibration line

| Concentration of L-lactic acid in [µg/ml] | Concentration of Glycolic acid in [µg/ml] | Slope *k* |
|---|---|---|
| 50 | 84.425 | 0.0007 |
| 75 | 126.64 | 0.0009 |
| 100 | 168.85 | 0.0009 |
| 150 | 254.28 | 0.0013 |
| 175 | 296.49 | 0.0015 |
| 200 | 337.70 | 0.0017 |

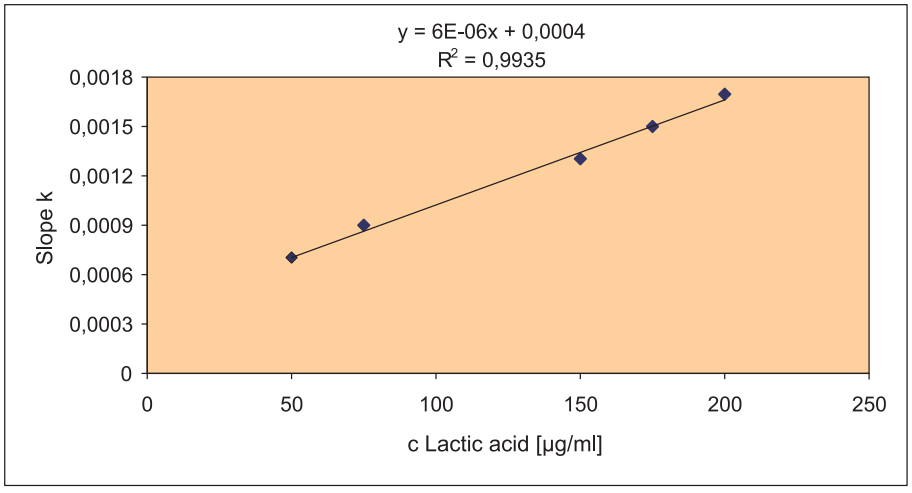


Figure 4 L-lactic acid-calibration line

the absorption of the samples was measured for 1 minute at 340 nm. The measured value was characterized as E1. As a blank 2 ml of Glycylglycine buffer pH 10 was used. Finally, the reaction was started by adding 1.12 U of L-lactate dehydrogenase to the cuvette with stock samples and the absorption was measured every 2.5 minutes over a period of 25 minutes. This measured value was characterized as E2.

For each measured concentration of stock dilutions a difference ΔE = E2 – E1 was calculated and plotted versus time in [min] and the corresponding slope k was determined.

Finally, the concentration of L-lactic acid was applied as a value of x-coordinate versus a corresponding slopes k as an ordinate values.

For generation of a calibration line a slope value for 100 µg/ml L-lactic acids was omitted, because of high deviation from other measured values as a result of influence of glycolic acid on enzymatic activity.

### *Quantification of L-lactic acid in PLGA polymer matrix*

#### Hydrolyzation of PLGA

For this purpose 200mg of Resomer 503H was diluted in 5 ml of 1 M NaOH and incubated over night (~16 hours) in a water bath at 35°C to accelerate hydrolysis of the polyester into its monomers (end concentration of PLGA 40 mg/ml). After incubation the hydrolyzed PLGA stock solution was diluted with distilled water to form PLGA samples in concentration range between 200 µg/ml and 550 µg/ml. Finally, the pH-value of each sample was adjusted with HCl to 10.

#### Test procedure

In order to correlate a ratio of L-lactic acid in PLGA after hydrolyzation with the theoretical ratio in the used Resomer RG 503 L-LDH activity was determined, and using a calibration line an amount of L-lactic acid for the following samples was calculated.

Table 4 Determinated values by quantification of L-lactic acid in PLGA polymer matrix

| Concentration of PLGA in [µg/ml] | Slope *k* | Calculated concentration of L-lactic acid in [µg/ml] |
|---|---|---|
| 200 | 0.0009 | 83.33 |
| 250 | 0.0007 | 50 |
| 300 | 0.0009 | 83.33 |
| 400 | 0.0011 | 116.67 |
| 500 | 0.0015 | 183.33 |
| 550 | 0,0016 | 200 |

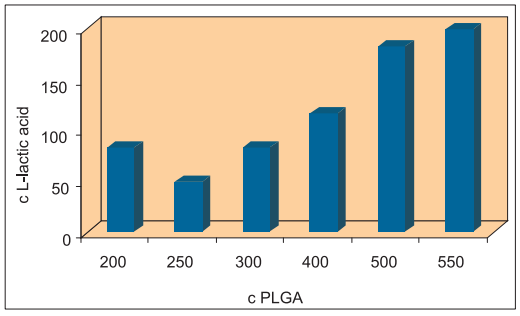


Figure 5 Correlation between concentration of PLGA and L-lactic acid

Figure 5 shows a linear correlation between the utilized PLGA concentration in the polymer matrix and the calculated concentration of L-lactic acid.

### *Quantification of L-lactic acid amount in purified PLGA nanoparticles*

To correlate the amount of L-lactic acid in purified PLGA nanoparticles with theoretical amount of L-lactic acid in PLGA polymer matrix which is frequently used for nanoparticles preparation, L-LDH activity and corresponding amounts of L-lactic acid was also determined for the following samples:

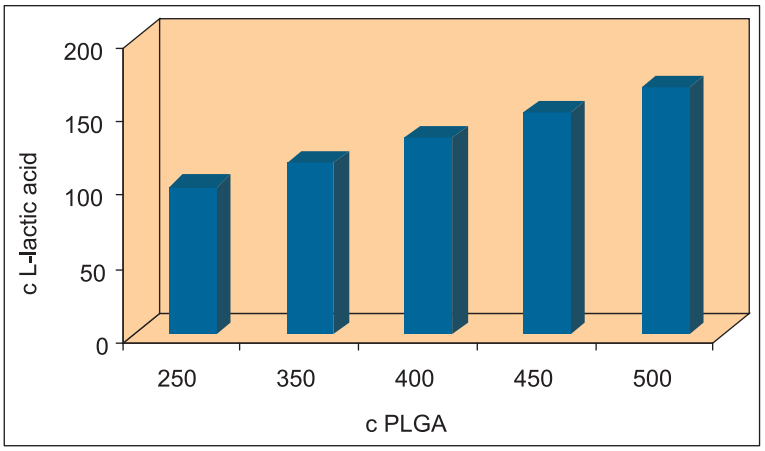


Figure 6 Correlation between concentration of PLGA and L-lactic acid in purified PLGA nanoparticles

Table 5 Determinated values by quantification of L-lactic acid in PLGA polymer matrix

| Concentration of PLGA in [µg/ml] | Slope *k* | Calculated concentration of L-lactic acid in [µg/ml] |
|---|---|---|
| 250 | 0.0010 | 100 |
| 350 | 0.0011 | 116.67 |
| 400 | 0.0012 | 133.33 |
| 450 | 0.0013 | 150 |
| 500 | 0.0014 | 166.67 |

Figure 6 shows that the calculated amount of L-lactic acid correlates with theoretical ratio of L-lactic acid in Resomer RG 503.

## Reaction with ferric (III) chloride

*Generation of a calibration line*

For corrected generation of calibration line we used a mixture of racemic D, L-lactic acid and glycolic acid in equal molar ratio 1:1, as is used Resomer RG 503.

A calibration line was generated by means of increasing D, L-lactic acid and glycolic acid amount. A stock containing 12 mg (0.13 mmol) D, L-lactic acid lithium salt (end concentration 600 µg/ml) and 10.128 mg (0.13 mmol) glycolic acid (end concentration 506.4 µg/ml) in 20 ml 0.01 M NaOH was prepared. Six dilutions of a stock in the range of 50 to 360 µg/ml of D, L-lactic acid and accordingly 42.2 to 303.8 µg/ml in 0. 01 M NaOH were used for generation of the calibration curve.

**Test procedure**

As a sensitive reaction indicator 0.1 g $FeCl_3$ * 6 $H_2O$ in 100 ml diluted HCl was used. In a first test step, 1 ml of stock dilutions (50-360 µg/ml D, L-lactic acid and 42.2 to 303.8 µg/ml glycolic acid) was reacted with 1 ml of $FeCl_3$ reagent. After incubation at room temperature (5 minutes) the absorption of each sample was measured for 1 minute at 360 nm and 370 nm. As a blank a mix of 1 ml $FeCl_3$ reagent and 1 ml 0.01 M NaOH was used.

Finally, the concentrations of D, L-lactic acid and glycolic acid were drawn as a value of x-coordinate versus a measured absorption as a ordinate values.

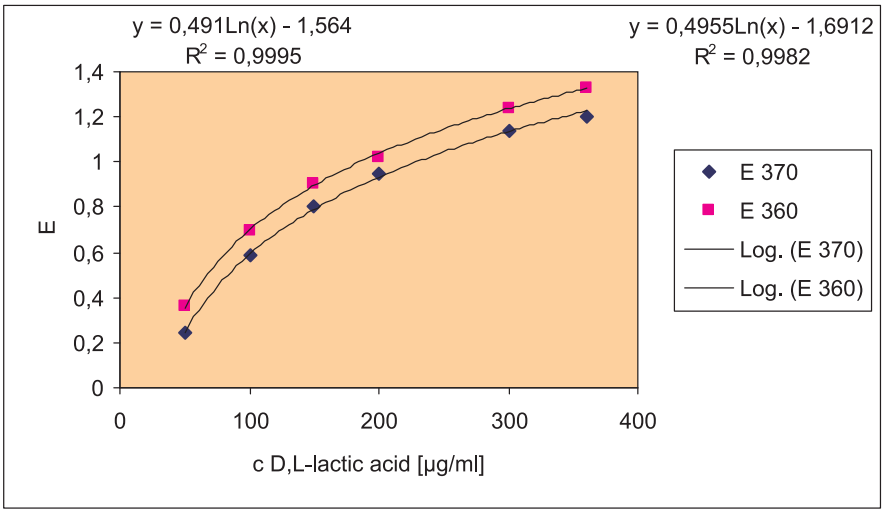


Figure 7 D, L-lactic acid-calibration line

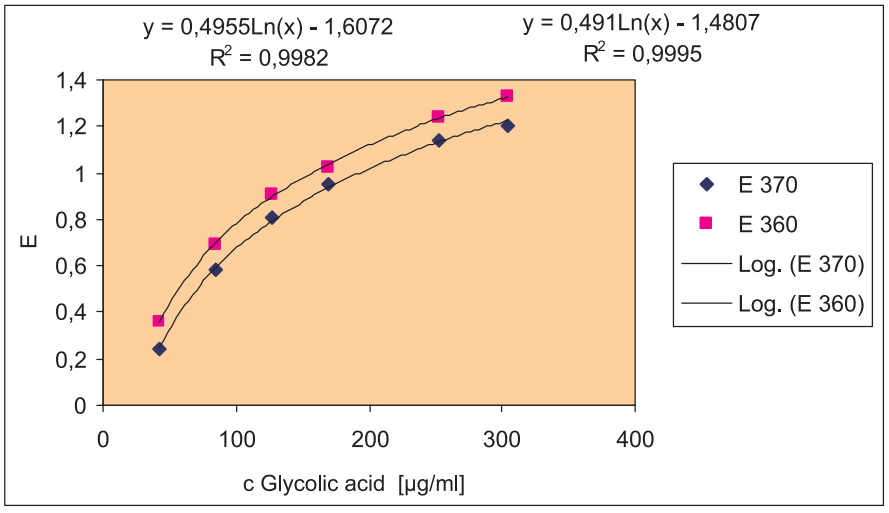


Figure 8 Glycolic acid-calibration line

*Quantification of D, L-lactic acid and glycolic acid in a PLGA polymer matrix*

The hydrolyzed PLGA stock solution was diluted with distilled water to form PLGA samples in a concentration range between 200 µg/ml and 600 µg/ml. 1 ml of PLGA dilutions (200-600 µg/ml) was reacted with 1 ml of $FeCl_3$ reagent. After incubation at room temperature (5 minutes) the absorption of each sample was measured for 1 minute at 360 nm and 370 nm.

A mix of 1 ml $FeCl_3$ reagent and 1 ml 0.01 M NaOH was used as a blank. Using a corresponding calibration line for D, L-lactic and glycolic acid the amount of both acids was calculated and compared to the concentrations of PLGA hydrolysates as given in the following tables.

Table 6 Results of $FeCl_3$ reaction for PLGA: measured values of samples at 360 nm and 370 nm: calculated concentration of D, L-lactic acid

| Concentration of PLGA [µg/ml] | Absorbance at 370 nm | Absorbance at 360 nm | Calculated concentration of D, L-lactic acid at 370 nm in [µg/ml] | Calculated concentration of D, L-lactic acid at 360 nm in [µg/ml] |
|---|---|---|---|---|
| 200 | 0.416 | 0.518 | 70.293 | 69.430 |
| 250 | 0.570 | 0.671 | 95.916 | 94.816 |
| 300 | 0.714 | 0.798 | 128.26 | 122.80 |
| 350 | 0.870 | 0.943 | 175.73 | 164.99 |
| 400 | 0.980 | 1.030 | 219.40 | 196.98 |
| 450 | 1.014 | 1.066 | 234.99 | 211.96 |
| 500 | 1.092 | 1.136 | 275.05 | 244.44 |
| 550 | 1.110 | 1.109 | 285.23 | 231.36 |
| 600 | 1.129 | 1.113 | 296.37 | 233.26 |

Table 7 Results of $FeCl_3$ reaction for PLGA: measured values of samples at 360 nm and 370 nm: calculated concentration of glycolic acid

| Concentration of PLGA [µg/ml] | Absorbance at 370 nm | Absorbance at 360 nm | Calculated concentration of glycolic acid at 370 nm in [µg/ml] | Calculated concentration of glycolic acid at 360 nm in [µg/ml] |
|---|---|---|---|---|
| 200 | 0.416 | 0.518 | 47.605 | 72.894 |
| 250 | 0.570 | 0.671 | 65.143 | 99.264 |
| 300 | 0.714 | 0.798 | 87.344 | 128.26 |
| 350 | 0.870 | 0.943 | 120.01 | 171.87 |
| 400 | 0.980 | 1.030 | 150.15 | 204.85 |
| 450 | 1.014 | 1.066 | 160.91 | 220.29 |
| 500 | 1.092 | 1.136 | 188.62 | 253.72 |
| 550 | 1.110 | 1.109 | 195.66 | 240.26 |
| 600 | 1.129 | 1.113 | 203.38 | 242.21 |

*Quantification of D, L-lactic acid amount in purified PLGA nanoparticles*

To compare the amount of D, L-lactic acid and glycolic acid in purified PLGA nanoparticles with theoretical amounts of both acids in PLGA polymer matrix which is frequently used for nanoparticles preparation, a test with $FeCl_3$ reagents was carried out and the corresponding amount of L-lactic acid and glycolic acid was also determined for the samples as shown in the following tables.

Table 8 Results of $FeCl_3$ reaction for PLGA nanoparticles: measured values of samples at 360 nm and 370 nm: calculated concentration of D, L-lactic acid

| Concentration of PLGA [µg/ml] | Absorbance at 370 nm | Absorbance at 360 nm | Calculated concentration of D, L-lactic acid at 370 nm in [µg/ml] | Calculated concentration of D, L-lactic acid at 360 nm in [µg/ml] |
|---|---|---|---|---|
| 200 | 0.251 | 0.333 | 50.384 | 47.634 |
| 250 | 0.436 | 0.516 | 73.188 | 69.148 |
| 300 | 0.640 | 0.716 | 110.47 | 103.92 |
| 350 | 0.688 | 0.775 | 121.71 | 117.18 |
| 400 | 0.818 | 0.893 | 158.22 | 149.02 |
| 450 | 1.004 | 1.063 | 230.29 | 210.67 |
| 500 | 1.103 | 1.162 | 281.22 | 257.74 |
| 550 | 1.152 | 1.191 | 310.46 | 273.42 |
| 600 | 1.186 | 1.212 | 332.51 | 285.36 |

Table 9 Results of $FeCl_3$ reaction for PLGA nanoparticles: measured values of samples at 360 nm and 370 nm: calculated concentration of glycolic acid

| Concentration of PLGA [µg/ml] | Absorbance at 370 nm | Absorbance at 360 nm | Calculated concentration of glycolic acid at 370 nm in [µg/ml] | Calculated concentration of glycolic acid at 360 nm in [µg/ml] |
|---|---|---|---|---|
| 200 | 0.251 | 0.333 | 34.018 | 50.181 |
| 250 | 0.436 | 0.516 | 49.584 | 72.600 |
| 300 | 0.640 | 0.716 | 75.124 | 108.70 |
| 350 | 0.688 | 0.775 | 82.839 | 122.45 |
| 400 | 0.818 | 0.893 | 107.95 | 155.37 |
| 450 | 1.004 | 1.063 | 157.67 | 218.96 |
| 500 | 1.103 | 1.162 | 192.89 | 267.39 |
| 550 | 1.152 | 1.191 | 213.13 | 283.50 |
| 600 | 1.186 | 1.212 | 228.41 | 295.78 |

## Discussion

*Determination of critical micelle concentration of anionic surfactant Pluronic F-68*

Pluronic F-68 was used for preparation of nanoparticles in a final concentration of 1.6%. Through degradation of nanoparticles with NaOH a dilution step of 1:100 was carried out, which results in Pluronic F-68 concentration of 0.16% in measured samples. Comparison of the concentration of Pluronic F-68 in samples (0.16%) with maintained critical micelle concentration (0.8%) suggests that Pluronic F-68 has an insignificant influence on the activity of lactate dehydrogenase.

*Optical test for quantification of L-lactic acid in PLGA polymer matrix and in purified PLGA nanoparticles*

Figures 5 and 6 show a linear correlation between the utilized PLGA concentration and the calculated concentration of L-lactic acid. From the obtained results a detection limit for quantification of L-lactic acid in PLGA polymer matrix could be defined in the range of 250µg PLGA/ml and 550 µg PLGA/ml and 250 – 500 µg/ml in PLGA nanoparticles.

## Reaction with ferric (III) chloride

*Quantification of D, L-lactic acid and glycolic acid in a PLGA polymer matrix*

According to the calculated values in Table 6, it can be deduced from the measured values at 370 nm, that the calculated concentration of D, L-lactic acid shows a higher correlation with the theoretical amount in Resomer RG 503. Dynamic range by this analytical method for D, L-lactic acid in polymer matrix is defined in a range between 300 µg/ml and 600 µg/ml. Due to the calculated concentration of glycolic acid as given in table 7, it is obvious that the measured values at 360 nm show a higher level of correlation with the theoretical amount of glycolic acid in Resomer RG 503. Dynamic range of quantification of glycolic acid in polymer matrix is defined in range of 250 µg/ml and 600 µg/ml. The light aberrances of measured results from the theory are caused by incomplete hydrolysis of PLGA.

*Quantification of D, L-lactic acid and glycolic acid in purified PLGA nanoparticles*

The results of ferric chloride reaction with the degradation products glycolic acid and D, L-lactic acid show a detection limit for lactic acid in PLGA nanoparticles in the range of 350 to 600 µg/ml. The detection limit for glycolic acid was 350 to 600 µg/ml in PLGA nanoparticles. The light aberrances of measured results from the theoretical values are a result of the purification process, during probably a small amount of polymer was washed out and the following incomplete hydrolysis of the nanoparticle-suspension.

## Conclusion

The results of the assay technique employing the ferric chloride reaction with the degradation products glycolic acid and D, L-lactic acid show a detection limit for lactic acid in PLGA between 300 – 600 µg/ml and in PLGA nanoparticles between 350 – 600 µg/ml. The detection limit for glycolic acid was 200 – 600 µg/ml in PLGA and 350 – 600 µg/ml in PLGA nanoparticles.

Concerning the optical test for assaying L-lactic acid the detection limit was 250 – 550 µg/ml in PLGA and 250 – 500 µg/ml in PLGA nanoparticles.

Beyond that it was possible in our work to show that glycolic acid acts also as a substrate for lactate dehydrogenase under the applied reaction conditions. But at the same time this molecule acts also as an inhibitor at the active site of this enzyme with respect to the substrate lactic acid. This makes the development of the optical test more complicated, since an appropriate correction for the calibration curve had to be established.

Both methods were evaluated and compared to each other. It was shown that an enzymatic assay is more sensitive but the ferric chloride method is simpler if both glycolic acid and D, L-lactic acid should be quantified simultaneously. During our work we were able to develop two sensitive methods for monitoring of biodegradation of polymers which are consecutively used as a nanoparticle matrix in drug targeting.

The enzymatic assay turned out to be more sensitive and should be given preference to the ferric chloride method if both glycolic acid and D, L-lactic acid should be quantified simultaneously. The ferric chloride method is by far the simpler method but severely prone to mistakes.

**Conflict of interest:** The authors declare that they have no conflict of interest. This study was not sponsored by any external organisation.

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