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Open Schools Journal for Open Science

Medical applications based on nanotechnology

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Abstract

Poly-lactic-co-glycolic acid can form nanoparticles which can be applied in nanomedicine as delivery vehicle for therapeutic agents. Here the aim is to reduce negative side-effects and to obtain higher local concentrations at the site of action. This work is about determining the binding-capacity of PLGA nanoparticles to several substances, applying a small set of model proteins. Our aim was to find out, whether PLGA nanoparticles can transport drugs in order to make drug targeting in medicine easier. This would bring a new era in medicine, with new therapy methods.

Keywords

Nanomedicine; controlled release; biocompatible; biodegradable; PLGA.

Acknowledgments

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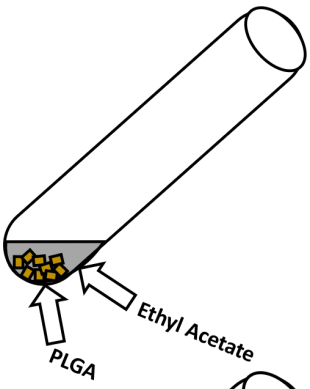
Introduction

Nanotechnology has become more and more important in many sectors from industry to medicine. Here, advances have been made in cancer therapy and diagnostics. Even a hybrid technology has been developed employing so-called theranostics, which can be used for diagnosis and therapy at the same time. Drug targeting is performed when specific markers guide the therapeutic vehicle directly to its action site in the human body. By such means, a much higher local dose can be reached and the side reactions are much less. Healthy tissue remains unaffected by this type of highly effective therapy.

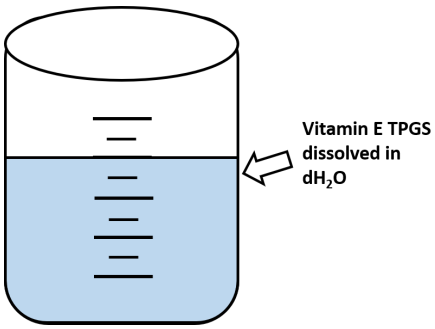
Poly-lactic-co-glycolic acid (PLGA) are polymers consisting of two different monomers, lactic acid and glycolic acid. As they are organic substances, they can be degraded by the human metabolism and they are, therefore, biocompatible. PLGA-NPs can be used in nanomedicine for the administration of drugs, in particular, for treatments based on biologics. These can be antibody treatments, such as immunotherapy in cancer. In my internship at the University of Salzburg we were using Vitamin E TPGS (0.3% & 0.9%) and PVA (Polyvinyl Alcohol) (0.3%), as emulsifier to optimize the process of PLGA nanoparticles' formation. Emulsifiers act to mix two substances, which are *per se* hard to mix. Without emulsifiers two phases, an organic and an aqueous phase, can be observed. Emulsifiers form very tiny droplets of one liquid substance in another one. The emulsion effects of the different experimental approaches which were tested in our study to optimize the PLGA-NPs were monitored by nanoparticle tracking analysis (NTA). Here, the aim was that rather small agglomerates were produced. As NTA uses light scattering, the term "monodispers" is used for a suspension which rather contains only one size of agglomerates. Different means, such as vortexing speed and ultrasonication, were used to find the best conditions yielding a PLGA-NP suspension with a low degree of polydispersity.

Synthesis of biodegradable PLGA nanoparticles by emulsion solvent evaporation



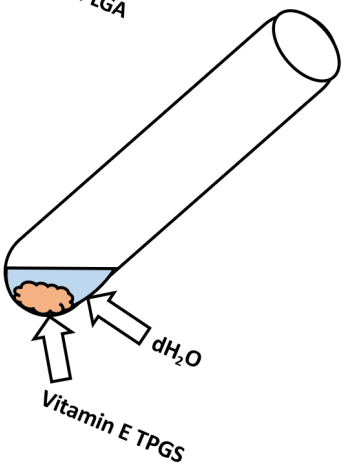
1) mix 100 mg PLGA with 1ml Ethyl Acetate (test tube I)

(on *Vortexer*)

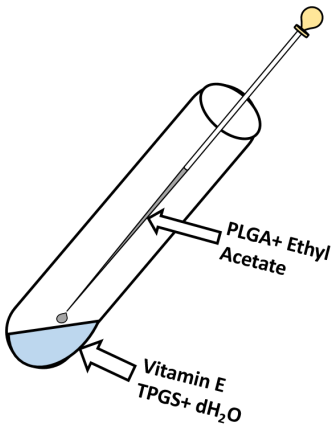


2) mix 0.3 g Vitamin E TPGS with 100 ml of distilled water (dH_2O)

(on *magnetic stirrer* until fully dissolved; use *stir bar*)



3) after it fully dissolves separate 45 ml of the liquid into a *beaker* and place it on the magnetic stirrer (360 rpm)



4) transfer 2 ml of the Vitamin E TPGS dissolved in dH_2O into a test tube (test tube II)

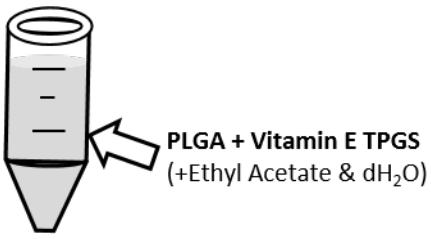
5) afterwards vortex test tube II and transfer the contents in test tube I to test tube II with a *Pasteur pipette*

6) use a *probe sonicator* to sonicate the final liquid of PLGA + Vitamin E TPGS. This results in a colour change from light white to milky white.

7) finally add the liquid from the *sonicator* to the 45 ml of Vitamin E TPGS (leave on magnetic stirrer for 3 hours)

8) after that, fill the fluid into Eppendorf tubes (2 ml) and put them into the *ultracentrifuge* (15 min; -6˚ C; highest speed 17000 g, two times)

9) after each centrifugation step, collect the supernatant without taking the particles which settled on the ground, with a pipette into a *Greiner* (50 ml). Refill Eppendorf tubes with water and use the ultracentrifuge for a second time with the same settings



10) fill particles mixed with water into a Greiner. Now there is a possibility of checking the size of the particles by making an NTA-test

Vortexing

Vitamin E TPGS

Centrifuge

Magnet stirrer

Size determination of PLGA particles

Four different solutions were investigated by NTA:

- PLGA nanoparticle + 0.3% w/v PVA (*speed*: 360 rpm, *time*: 4 h, *dilution*: 1:100)
  - larger size (184.9 nm)
  - very polydispers
  - 7.14e+008 +/- 2.95e+008 particle/ml
- PLGA nanoparticle + 0.3% w/v Vitamin E TPGS (*speed*: 360 rpm, *time*: 4 h, *dilution*: 1:100)
  - size ~ 275.9 nm
  - polydispers – but less than with PVA
  - 2.00e+009 +/- 7.98e+007 particle/ml
- PLGA nanoparticle + 0.9% w/v Vitamin E TPGS (*speed*: 360 rpm, *time*: 4 h, *dilution*: 1:100)
  - size ~216.9 nm
  - most monodispers from all examples
  - 2.54e+009 +/- 6.19e+007 particle/ml
- PLGA nanoparticle + 0.9% w/v Vitamin E TPGS (*speed*: **1100 rpm**, *time*: 4 h, *dilution*: 1:100)
  - size ~ 214.4 nm
  - low polydispersity
  - 2.47e+009 +/- 6.53e+007 particle/ml

In summary, the biggest difference was observed between 0.3% w/v PVA and 0.3% w/v Vitamin E TPGS solutions. The difference between 0.3 and 0.9% w/v Vitamin E TPGS at 360 rpm centrifugation speed was not as big as with PVA. Different centrifugation speeds (360 and 1100 rpm) do not differ for Vitamin E solutions. We saw that PVA and Vitamin E have an influence on dispersity and size of the resulting NPs, concentration has an influence too, as well as the centrifugation speed. The size of the PLGA-NPs was not reduced by ultrasonication, which was surprising.

Gel electrophoresis of PLGA-bound protein

Some of the proteins mix with the nanoparticles and bind to them. In order to find out the real amount of proteins which bound to the particles, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is needed. The process is divided in two parts: the first one is separating the proteins by their size in the SDS-Gel and afterwards, coloring the Gel with Coomassie R250 to determine the amount of the proteins bound with the particles. The second part is quantifying the unbound proteins in the supernatant by using Bradford Assay. Having finished both parts, the quantity of the proteins bound to PLGA-nanoparticles can be determined.

Preparation of samples for SDS-PAGE

1) The nanoparticles are filled into Eppendorf Tubes (100 µg/ml) and mixed with proteins (20,40 µg/ml), lysozyme (LYZ) or *bovine serum albumin* (BSA). In addition to that, the "eppis" must be pinned on a Tube Rotator overnight (20-24 h). During this time, the proteins will ideally bound to the nanoparticles and build a Bio Corona around themselves.
2) Now the liquids need to be centrifuged (5000 g, 5 h, 4˚C) so that the bound particles settle down. Afterwards, the supernatant including the unbound proteins is removed and filled into other eppis (for each LYZ & BSA 3 eppis, 10/20/40 µl). They will be used in the second part of the experiment.
3) Preparing our standardized samples in order to compare the bound particles to them is the next step. If the concentration of our standardized samples is known, the amount of the bound proteins can be guessed. For our standardized samples we used the following concentrations:
   - 40 µg/ml
   - 20 µg/ml
   - 10 µg/ml
   - 5 µg/ml

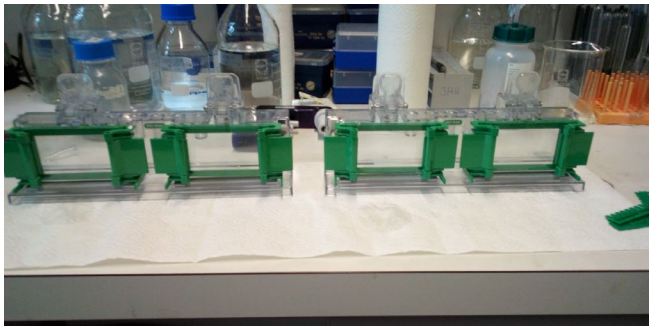
4) For our samples, we need to mix each Lysozyme and BSA eppi with 12µl water and 4 µl denaturing buffer, heat them to 95˚C for 10 minutes and centrifuge shortly. The reducing agent includes:
   - SDS (breaks complex protein structures into a linear chain of amino acids)
   - β-mercaptoethanol (breaks the strong di-sulfide bonds in the proteins)
   - Tris Chloride
   - Glycerol (makes proteins foreseeable while putting them into the stacking gel)
   - Water
   - Bromphenol blue (makes proteins foreseeable while putting them into the stacking gel)
5) In order to find out the amount of the unbound proteins, standardized samples in the same concentration as used before, are necessary. To get these, 40 µl sample is mixed with 160 µl Bradford reagent. Thus, a dilution of 1:4 is achieved. As soon as the samples are mixed with Bradford reagent, the colour of the sample changes from reddish brown to blue in the presence of protein. The intensity of the colour determines the amount of the proteins and it is measured as absorbance with a Plate Reader. Below, the reducing agent for breaking the disulfide bridges and a microtiter plate used for photometric measurements are shown.

Conducting SDS-PAGE

1) Two gels, a stacking gel and a separating gel, are used to determine the quantity of the bound particles. The separating gel, which must be produced firstly as it has to be placed under the stacking gel, separates the bound molecules by their size. It includes:
   - 7,5 ml H_2O
   - 7,5 ml loading buffer
   - 15 ml acrylamide
   - 15 µl TEMED
   - 90 µl Aps

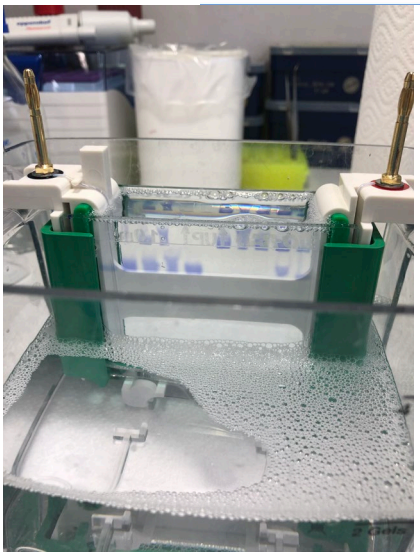
2) Next step is producing the stacking gel. This is prepared with the following components:
   - 5,2 ml H_2O
   - 2,2 ml upper buffer
   - 1,4 ml acrylamide
   - 14 µl TEMED

- 100 µl APS

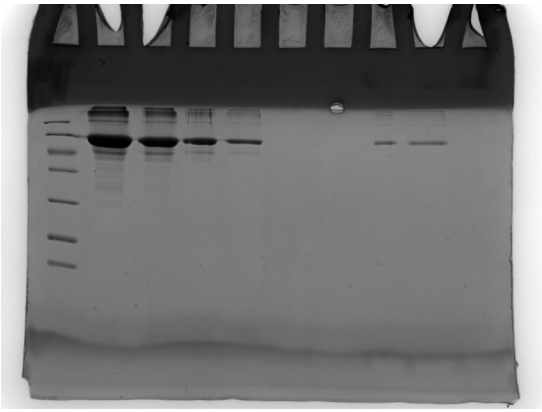


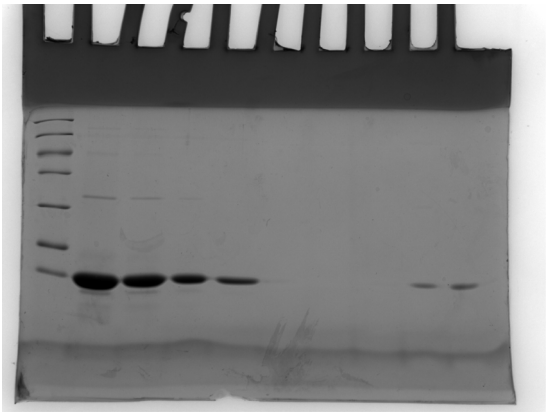
In order to form the wells of the gel a comb is needed, which must be placed into the device after filling in the still liquid stacking gel on top of the separating gel as depicted at the right side.

3) Afterwards, the two prepared gels are placed oppositely into the chamber, which is filled with the running buffer . Having done that, the device must be connected to power cables and therefore to the voltage source as well (0.06 A for 45 min) so that one end has a positive charge and the other end a negative charge. In this electric field, the proteins will be separated by their size. Generally, using an electric field to influence an object's motion is called ***electrophoresis***. The more concentrated the gel is, the slower the proteins will traverse through it. Small proteins travel more quickly and therefore migrate further through the gel.
   After some time, the proteins travel down based on their size. The amount of proteins present can be determined from the bands developed. These bands can be compared to the standardized samples and therefore, the amount of proteins can be calculated.
4) Having removed the gels from the device, they should be put in Coomassie Blue for 30 min so that the proteins get an intensive color and can be better seen. After that, the gels should be kept in destaining buffer for some time to reduce the background staining.
5) To determine the exact amount, an image is taken using a digital camera. The image is further evaluated using a specific software (ImageLab). Below, the gel after running with blue marker depicting the gel slots is shown on the left. At the right side the staining procedure is shown.



Below the two SDS-PAGE gels of BSA (left side) and lysozyme (right side) are shown. The first lane of each gel shows the protein size marker verifying the molecular weight of the proteins. The subsequent four bands indicate different concentrations of protein standards allowing quantification by comparison. In the last two lanes show the two respective nanoparticle-bound proteins, BSA and lysozyme.





Conclusions

Using this procedure, we were able to quantify the amount of nanoparticle-bound proteins. However, the binding efficiency was very low. It was thus concluded, that further alternative procedures, like e.g. encapsulation of protein into the PLGA nanoparticles, could be adopted to improve protein loading.