Nanoparticle production guide

Introduction

Binanox created this step-by-step guide to help you safely create nanoparticles. We talk about every aspect that you need to take into consideration, when working with nanoparticles. It will guide you through the choice of organism, choice of metal, what material is needed, and what safety precautions you need to take.

Step 1: Pick your fighter

Science

For green biosynthesis of nanoparticles, a wide selection of organisms may be used. These include bacteria, fungi and even plants. However, all of them create different kinds of nanoparticles, therefore it is first important to ask yourself certain questions to know which organism you should choose:

1. What material should I use ?

2. What size and morphology should they have?

3. What organism can produce these specific nanoparticles?

In the Binanox project we mostly focused on the medical application of nanoparticles, as we want to use them in photothermal therapy as a head and neck cancer treatment.

1.Material

Nanoparticles can be made from a variety of materials, but the choice of material used is largely determined by the final purpose of these nanoparticles. Given that we focused on photothermal therapy using metallic nanoparticles, we largely focused on the potential of silver and gold during our project.

Silver

Silver nanomaterials are also suitable candidates for PTT, due to their low toxicity, easy production, anticancer properties and excellent heat conductivity[1]. Additionally, these particles are extensively used as novel antimicrobial agents, detection and diagnostic platforms, tissue restoration materials, and personal healthcare products. To produce silver nanoparticles in our project we used the salt silver nitrate (AgNO3).

Gold

The most explored metallic nanomaterials for PTT are gold nanomaterials, due to enhanced absorption, excellent photostability, tunable physical and optical properties, high biocompatibility, and great heating effect[1]. Additionally, gold nanoparticles have a high efficiency in light-to-heat conversion, due to their surface plasmon resonance[2]. These particles have also been used for diagnostics[3]. In our project, we used the salt gold chloride (HAuCl4) for the formation of golden spikes.

Given the advantages of these metals, we aimed to use both silver and gold to create bimetallic nanoparticles with a silver core and golden spikes. This enables us to combine the advantageous properties of these metals to enhance the overall properties of the nanoparticle for PTT.

2.Size and morphology

Nanoparticle size influences the suitability of the nanoparticles for PTT. Thus, we needed particles smaller than 200 nm, to avoid immediate clearance from the body, which would render the therapy ineffective4. Furthermore, you want to avoid accumulation of nanoparticles in certain organs. Studies on mice were conducted to find out what size leads to the least accumulation, they came to the conclusion that particles with a size of 50 nm accumulated the least compared to all other sizes[5]. Therefore, if you want to use nanoparticles for PTT you should aim for a size of 50 nm.

The morphology is another critical factor to consider while producing nanoparticles for PTT. Nanoparticles come in all different kinds of shapes and each of them has its advantages and disadvantages. Nanorods, nanoshells, and nanocages have hollow centers and can for example be used for drug delivery[2].

Nanotriangles have a plasmonic resonance in the NIR region making them suitable for photothermal therapy[1]. Spherical nanoparticles have an absorption range between 500 and 600nm, which is in the visible spectrum. However, changing the shape to a non-spherical one can shift the absorption to the NIR region, which ranges between 750 and 900 nm6.

Similarly, nanostars, branched nanoparticles, or urchin-like nanoparticles show absorption in the NIR region. They consist of a core with sharp tips, where the size of the tips influences the optical properties[7]. Applied electrical fields can be enhanced near the tips of the stars, leading to heat generation. Therefore, these nanoparticles may be suitable for effective photothermal ablation8. The urchin-like nanoparticle was chosen for the Binanox project as it is suitable to convert a NIR spectra into heat for effective tumor ablation.

In the Binanox project we aimed to create nanoparticles of 50 nm that have an NIR region of around 800 nm.

3.Organism

Fungi

Extracts of fungi are excellent candidates for the synthesis of metal nanoparticles, due to the scalability and cost efficiency of fungal growth. Filamentous fungi can be used to produce a variety of metal nanoparticles, including gold, silver, iron oxide, and bimetallic ones. The sizes range from 6-40 nm and so far mostly spherical and hexagonal shapes were created[9].

Plants

Plants contain biomolecules with the potential to reduce metal salts into nanoparticles. Various plants such as aloe vera, oat, coriander, and lemon have been used to synthesize mainly silver and gold nanoparticles. However, mustard, alfalfa, and sunflowers can also produce zinc, nickel, cobalt, and copper nanoparticles. So far, mainly spherical and hexagonal shapes were observed. The sizes range from 2-35 nm[10].

Bacteria

The type of bacterial species you would use for nanoparticle synthesis will highly influence your final product.[11] Several microorganisms are known to trap metals in situ and convert them into elemental nanoparticle forms. Other reports suggest that nanotization is a way of stress response and biodefense mechanism for the microbe, which involves metal excretion/accumulation across membranes, enzymatic action, efflux pump systems, binding at peptides, and precipitation.[11] The most explored strains for silver nanoparticle production

are among others *Escherichia coli*, *Lactobacillus casei*, *Pseudomonas proteolytica*, or *Enterobacter cloacae*. For gold nanoparticles the most common strains are E. coli DH5α, *Bacillus subtilis*, or *Shewanella alga*. The sizes range from 2-100 nm.

4. Genes

Moreover, genes also play an important role for microbial nanoparticle biosynthesis. For the choice of genes it is important to consider if they are membrane bound or present in the periplasmic space. Another important factor is the choice of promoter and the inducible system. Understanding the molecular mechanism also allows us to control the shape, size, and monodispersity of the NPs to develop large-scale production according to the required application[11].

What kind of genes will you incorporate into your GMO? For instance, you could enhance the resistance to heavy metals or optimize a silver reduction pathway. In our project we chose to engineer bacteria to make them less susceptible to metal stress by transforming genes from the metal resistant bacteria *Cupriavidus metallidurans*.

Step 2: Safety before entering the lab

1. Talk to the safety people from your university

After you have chosen your material, organism and genes you need to talk to the responsible safety people from your university before entering the lab. Working with nanoparticles requires special safety measures and they can help you assess those. They made our team fill out a safety assessment sheet, where it was calculated what potential risk our nanoparticles pose and what safety measures needed to be taken.

2. Lab biosafety level

There are four Risk groups with 4 being the highest. These levels of safety are also important when working with GMOs to ensure that they do not escape from the laboratory as their risk to nature is likely unknown. Before conducting experiments involving the construction of GMOs a biosafety risk assessment should be conducted together with experts from the university or governmental institutions. There are certain biosafety considerations for expression vectors

that need to be taken into account. Working with a well known plasmid that has been completely sequenced in combination with for example *E. coli*, poses little risk and can safely be performed in a Level 1 lab. The table below summarizes the biosafety levels given by the World health organization[12]:

Biosafety level	Level 1	Level 2	Level 3	Level 4
Description	•No containment •Defined organisms •Unlikely to cause disease	•Containment •Moderate Risk •Disease of varying severity	•High containment •Aerosol transmission •Serious, potential lethal disease	•Maximum containment •High-Risk agents •Life-threatening disease
Sample organism	*E. coli*	Influenza, HIV	Tuberculosis	Ebola
Pathogen type	Minimal potential hazard to the environment	Agents associated with human disease & pose moderate risk to the environment	Agents presenting a potential risk for aerosol transmission & causing serious or potential lethal disease	Agents that pose a high risk of aerosol transmission & life-threatening disease

2. Personal protective equipment needed for work with nanoparticles

The wear of protective equipment is based on the Hazard Statements or H-codes given to chemicals. During the Binanox project we were using silver nitrate ($AgNO_3$) and chloroauric acid ($HAuCl_4$) which pose the following risk:

Silver nitrate		**Chloroauric acid**	
H sentence	**Description**	**H sentence**	**Description**
H272	May intensify fire; oxidizer	H290	May be corrosive to metals
H290	May be corrosive to metals	H302	Harmful if swallowed
H314	Causes severe skin burns and eye damage	H314	Causes severe skin burns and eye damage
H410	Very toxic to aquatic life with long lasting effects	H373	May cause damage to organs through prolonged or repeated exposure
		H411	Toxic to aquatic life with long lasting effects

Based on this we were advised to use the following PPE:

· Wear a lab coat with long sleeves

· Wear nitrile gloves over the sleeves of the lab coat

· Protective glasses

· If nanoparticles are not kept in solution, use respiratory protection and work in a ventilated system (i.e. fumehood or biosafety cabinet)

4. Equipment needed for work with nanoparticles

In general, it is important to understand that nanoparticles spread like gasses and most harm is done when inhaled. Therefore, it is important to prevent nanomaterials from becoming airborne and spreading into the environment. An important safe working method is to keep the nanomaterials either in solution or bound to a matrix and to work in a closed system as much as possible, or at least in a well ventilated system (i.e. fumehood or biosafety cabinet class 2 (HEPA filters, filter nanomaterials out too). Also, pay attention to processes in which solutions are heated and/or centrifuged/mixed/resuspended as this often creates aerosols that can increase possible exposure. Also, formation or use of (fine) powders should be prevented as much as possible. We were advised the following safety measures:

- When working with GMO's only (*E.coli*, ML-1). Work can be performed on the lab table using safe microbiological working methods.
- When working with the nanoparticles only, work in a fumehood or biosafety cabinet class 2 with HEPA filter.
- When working with both nanoparticles and GMO's, work in a biosafety cabinet. Even better is to use a biosafety cabinet that ventilates to the outdoors.

Step 3: Creating your perfect nanoparticle

1. Growth conditions

If you opted for bacteria, the growth conditions are highly important to obtain the optimal yield, shape, and size. In the Binanox project we did a great number of optimization experiments to produce the perfect nanoparticles for PTT. All the experiments that were done can be found in the notebook and we created a model predicting the perfect conditions.

2. Cell or cell-free system?

Now that we have obtained our GMO for optimal nanoparticle synthesis, we have to determine how long we will use this GMO in the production pipeline. We will discuss the possible upsides and downsides of using a cell system synthesis approach or of a cell-free synthesis approach.

Cell system

A big advantage of a production pipeline with a cell system is that it can, if provided by the necessary resources, keep a continuous nanoparticle production. However, proper purification of the nanoparticles from the cell system while making sure that there are no GMO contaminations can be a big challenge.

Possible problems with a cell-free system could be: The machines that are used for extracting could be contaminated by GMOs. In some cases, completely preventing this risk is not possible at all. For example if the end product is a medical application, the use of GMOs might be a problem. Due to the problems that one could encounter when trying to extract the nanoparticles from a cell system we would recommend minimizing the use of a cell system, especially in an iGEM project.

Cell-free system

There are some examples of why you would want to make use of a cell-free system in an iGEM project, for instance, some recommendations are:

- No DNA mutation of your genetically engineered device because there is no DNA replication
- No selective pressure on your genetically engineered device because the system is non-living and does not undergo natural selection
- No self-replication of your genetically engineered device leads to a fixed amount of DNA being expressed and more control over the rate of expression
- Expression system can be quality-controlled by manipulating adjustable parameters[10]

During your iGEM project you can also partially use a cell system and switch over to a cell-free system later. You will find out below that this is an approach that we used.

Our Binanox nanoparticles are intended to be used for medical therapy, that's why it is of the utmost importance that the nanoparticles are free from every possible infectious source (See the interview with Aaike van Vught CEO Vsparticle on our human practices). We decided to use bacteria that overexpress and secrete proteins in the medium, which was subsequently filter-sterilized and used for nanoparticle synthesis. This approach is cell-free and not only does this reduce the potential infectious hazards of our final product it is also helpful for the extraction step so that you have more options due to not working with GMOs.

Step 4: Safety in the lab

1. Waste & Spills

It is important to have waste management in the lab and to know exactly what for example goes in the GMO waste and what goes in the chemical waste.

- Waste with GMOs can first be treated with Ethanol 70%, bleach or autoclaved for liquids waste to kill the bacteria. Then liquid waste can be thrown away in the liquid waste cans
- Liquid (small) nanoparticle spills can be collected with a tissue in a local plastic bag and the bag can be discarded in the solid chemical waste containers. If the spill also contains GMO bacteria, ethanol 70% has to be used.
- Nanoparticle spills/powders can be collected with a wet tissue and thrown away into the solid chemical waste containers (make sure you move slowly, to prevent particles from getting airborne).

2. Transport

Sometimes you might need to transport the nanoparticles outside of the lab, for example to perform certain measurements. It is important that this is done in a safe way, to prevent any exposure to the environment.
When transporting samples with nanoparticles outside the lab, always use a double containment system (i.e. Eppendorf tube in sealed plastic bag). This to prevent the solution from spreading (into the environment) if, in case of an incident, the tube is broken.

Step 5: Extract your nanoparticle

Before we can start the extraction of the synthesized nanoparticles, we have to make sure it is completely free of GMOs. The sterilization processes of nanoparticles by autoclaving and filtration are two of the most utilized methods in the pharmaceutical industry but are not always viable options[13]. Autoclaving is highly discouraged, as the high temperatures of autoclaving could possibly change the morphology of the nanoparticles, causing a change in absorbance and thermal conductive potential. Highly effective and low-processing-time options for sterilizing nanoparticles for medical purposes are Gamma-radiation and UV-radiation[13].
After sterilization various extraction methods can be chosen, they all have certain pros and cons. Some extraction suggestions are:

Field flow fractionation

Field flow fractionation (FFF) is a conventional method that employs several external force fields. A field is considered effective if its strength and selectivity are sufficient to achieve separation. Typical fields include cross-flow streams, temperature gradients, electrical potential gradients, centrifugal force, dielectrophoretic force, and magnetic force[14].
This can be a powerful technique to separate different types of nanoparticles.

Ultracentrifugation

Another method that can be used for nanoparticle extraction and separation is ultracentrifugation. In this method, a sample is spun at an extremely high G-force, which makes it possible to separate components of the sample in a gradient. With this method, it is also possible to separate nanoparticles by size. This can be done with a relatively simple sucrose gradient[14]. See used protocol[15].
The synthesized nanoparticles will not all be homogeneous, which is why this method can be extremely helpful. One can make a gradient and extract the different fractions. In this way it is possible to only extract nanoparticles of a certain desired size.
There are a few downsides to this method: Again, an expensive (ultracentrifugation) machine is required for this methodology. Additionally, this method is only useful if you want to extract small amounts of samples since it would be inefficient to use it on a bigger scale. Safety-wise this method is not ideal because ultracentrifugation of the sample will generate aerosols. To avoid contact with aerosols you need to wait at least an hour before it is possible to work with the samples.

Size exclusion chromatography

SEC (size exclusion chromatography) is a column liquid chromatographic technique commonly used for the separation of macromolecules in solution. Typically, SEC columns are packed with small, rigid porous particles of sizes ranging from 3 to 20 μm and pore sizes from 50 to 107 Å. SEC separates molecules according to their size in solution or, more specifically, their hydrodynamic volume. The larger molecules in a sample elute before the smaller molecules because larger molecules either enter fewer pores or sample a smaller pore volume of the column packing material (depending on whether the column is of a mixed-bed or individual pore size) than their smaller counterparts[16].
This is a very promising technique to use for nanoparticle extraction because one can design columns specifically to suit the extraction of the desired nanoparticles. In literature you can find a variety of eluents, columns and detection methods to suit one's needs. There is a

significant challenge in the SEC analysis of metal nanoparticles in measuring their adsorption to the column packing material. This means that part of the sample will remain inside the small rigid porous particles. Adsorption can cause several problems in the SEC analysis of nanoparticles.[16]

Secondly, buying or constructing these columns can be an expensive task. And these expensive columns can only be reused for a limited amount of time, so keep this in mind when you choose this as a potential extraction method.

We tried the ultracentrifugation extraction method. To keep the risk of aerosol exposure to a minimum, we waited an hour after the ultracentrifugation was done before opening the tubes. This made the process for ultracentrifugation extraction almost twice as long. However, we highly advise other iGEM teams to do the same. Nanoparticle exposure through aerosols is one of the most dangerous ways to get exposed[17]. See the Binanox result section for the outcome of this extraction method.

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