Synthesis of drug loaded magnetic nanoparticles and their uptake into immune cells

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Abstract. Ferrite nanoparticles (Mn0.8Zn0.2Fe2O4) are synthesized by the co-precipitation method and characterized by X-ray diffraction, transmission electron microscopy and dynamic light scattering. The particles are functionalized with dextran which is activated via amino or carboxymethyl groups. The chemotherapeutic drug doxorubicin (DOX) is attached to these dextran derivates in different ways. One method is based on the attachment of DOX to amino dextran by its keto group; the other is a bond to the primary amino group of DOX. The characterization of drug loaded dextran derivates is performed by Raman, FT-IR-, UV/VIS- and fluorescence spectroscopy. The biofunctionalized particles are intended for use in adoptive cancer immunotherapy as a new approach, where immune cells (T lymphocytes) will be used as new autonomous highly target specific drug delivery systems. The uptake efficiency of these particles into T lymphocytes is investigated by fluorescence and convocal microscopy.

1. Introduction

Magnetic nanoparticles (NPs) with different surface modifications like citrate, dextran / starch and poly-ethylene glycol, etc. have been used experimentally for several in vitro and in vivo applications such as magnetic resonance imaging (as contrast agents)[1], hyperthermia[2], drug delivery[3, 4], immunoassay[5], magnetic cell separation and for the delivery of gens[6], enzymes or DNA[7, 8]. For biomedical applications, it is necessary to coat the surface of the nanoparticles with a special, non-toxic and biocompatible material. It is important, especially for in vivo applications, that all these nanoparticles are non-toxic and do not react with the biological system. The biofunctionalized magnetic nanoparticles can be directed to an organ, tumour or tissue using an external magnet or can be heated in alternating magnetic fields for controlled drug release or used in hyperthermia. Controlled drug release implies the ability of the regional and temporal distribution of the active drugs in the body. Accordingly to this the aim is to concentrate the drug in the involved tumour region in a high therapeutic-optimal range and to stay under the toxicity barrier in the rest of the body. Therefore the cumulative dose could be reduced and the side effects decreased.

So the aim of this project is the magnetic controlled drug-positioning of located, drug loaded magnetic nanoparticles in immune cells (especially T lymphocytes) and a magnetically controlled drug release. T-lymphocytes are intended for use in adoptive cancer immunotherapy. A new approach where immune cells will be used as new autonomous highly target specific drug delivery systems. Due to his auto-fluorescence Doxorubicin is used as a model chemotherapeutic drug.
2. Experimental

2.1. Ferrofluid
Substituted nanoparticles (NPs) with the composition Mn$_{0.8}$Zn$_{0.2}$Fe$_2$O$_4$ are synthesized by the chemical co-precipitation method $^{[9,10]}$. For biomedical application it is necessary to use non-toxic metal ions and to stabilize the NP at a pH value of 7, for this purpose citrate is used. The particles synthesized in this way are characterized by X-ray diffraction (XRD), transmission electron microscopy (TEM) and dynamic light scattering (DLS). The size distributions of the particles are shown in Fig. 1.

![Figure 1](image1.png)

**Figure 1.** Left: Distribution of hydrodynamic diameters of the different stabilized NPs; Right: X-ray diffraction of the typical reflexes of Mn$_{0.8}$Zn$_{0.2}$Fe$_2$O$_4$ compared to the JCPDS database, crystallite size (estimated via Debye-Scherrer formula) is between 10-15nm.

2.2. Synthesis of DOX-CMD coated magnetic nanoparticles
Carboxymethyl dextran (CMD) is synthesized by esterification of the hydroxyl groups via monochloro acetic acid, sodium hydroxide solution and iso-propanol. A higher degree of substitution (DS) can be obtained by repeating the given synthesis. Now the NPs were coated under stirring and heating conditions with the synthesized CMD. The attachment of the primary amino group of doxorubicin to the CMD coated magnetic NPs leads to an acid amid bond in the present of the crosslinker 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS).

2.3. Synthesis of DOX-Hyd-CMD coated magnetic nanoparticles
Hydrazine-CMD derivate (Hyd-CMD) is synthesized by using hydrazine, CMD and EDC. The coating of the NPs with Hyd-CMD is the same procedure as CMD coating. The bonding of the drug to Hyd-CMD is done by a reductive amination, where the carbonyl group of the drug reacts with the amino group of Hyd-CMD, via an intermediate. The intermediate hydrazone is reduced in the presence of sodium cyanoborohydride (NaBH$_3$CN). A catalytic amount of acetic acid is used, too.

3. Results and discussion
Due to the fact that the polymer coating on the particles surface is difficult to observe by TEM, because there is no significant difference between the morphology of the uncoated particles and the coated ones, DLS is used to estimate the hydrodynamic diameters of the coated particles. The results of the DLS measurements are presented in Fig. 2. The particle size distribution (polydispersity $\sigma$) has to be between one and two to have a well dispersed system with a low agglomeration. All coated NPs show a low agglomeration degree, which can be seen in the TEM pictures too. The agglomeration degree plays an important role for biological applications because of the embolism risks. Fluorescence correlation spectroscopy (FCS) is also used to prove the surface coating of the NPs. FCS shows that the diffusion coefficient of the free Doxorubicin is significantly lower than the diffusion coefficient of drug loaded nanoparticles.
The isoelectric point of the coated NPs is below pH 7. This is necessary for medical application, because this guarantees the stability of the particle at the physiological pH.

Figure 2. Left: Distribution of hydrodynamic diameters of the Hyd-CMD/DOX-Hyd-CMD coated NPs; Right: Distribution of hydrodynamic diameters of CMD/DOX-CMD coated NPs.

To prove the coupling of the drug to the functionalized nanoparticles we use FT-IR spectroscopy. Fig. 3 shows the typical FT-IR spectra of the different surface modified NPs. At wave number ~1750 cm\(^{-1}\) the characteristic carbonyl stretching vibration is shown. The other bands are correlated to the vibrations of dextran. Due to the bonding of the amino group of DOX to CMD and hydrazine to CMD, respectively, a shifting in the carbonyl vibration peak takes place.

Figure 3. Left: FT-IR spectrum of Hyd-CMD/DOX-Hyd-CMD coated NPs; Right: FT-IR spectrum of CMD/DOX-CMD coated NPs.

The uptake efficiency of these particles into T lymphocytes is investigated by TEM measurements, fluorescence and convocal microscopy, and by fluorescence activated cell sorting (FACS). The TEMicrographs (left and right) in Fig. 4 show the uptake of the DOX-CMD functionalized NPs in T cells probably via endocytotic vesicles.

Figure 4. TEMicrographs of activated T cells after incubation with 20µg/mL DOX-CMD loaded nanoparticles for about 2h at 37°C.
Another method to prove the uptake of drug loaded NPs into T cells is done with FACS. This analysis also shows the uptake of the NPs due to the increase of the fluorescence intensity of loaded T cells with DOX.

Fig. 5 shows the fluorescence microscopy pictures of DOX-CMD loaded NPs in T lymphocytes. The pictures (left and right) are made by a band gap filter of red light and show several spots in the cell. This is supposed to be a phase of the apoptosis, in which the nucleus fragments.

Figure 5. Fluorescence microscopy of DOX-CMD loaded NPs T lymphocytes, T lymphocytes are marked with CD45-FITC; T cells after incubation with 20µg/mL Doxorubicin-CMD nanoparticles for about 20h at 37°C.

4. Conclusions
We have found two different ways to bond the drug to the magnetic NPs. In the first method the drug bonds to the carboxyl groups of CMD-coated nanoparticles via acid amide formation. The other method leads to a bonding between Hyd-CMD coated NPs and keto group of the drug via reductive amination. It has been shown that the drug loaded NPs are internalized by the cells via TEM- and fluorescence microscopy measurements. TEM pictures on the uptake behaviour of drug functionalized NPs by T lymphocytes have demonstrated that the DOX-CMD-coated NPs are taken up by the cells and clustered in membrane-surrounded cellular structure (endocytotic vesicles). The measurements for the uptake of DOX-Hyd-CMD coated NPs into T cells are still running. So far we can only present the effects of DOX-CMD-coated NPs on T cells.

Further the drug-positioning of located, drug loaded magnetic NPs in immune cells and the controlled release of the drug in the tumour region is planned. Another study is the encapsulation of the drug loaded NPs for a better survival rate of the T lymphocytes.

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