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Modelling the response surface to predict the hydrodynamic diameters of theranostic magnetic siRNA nanovectors

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ABSTRACT

Short interfering RNAs (siRNAs) appear to be a promising tool to treat various human diseases, such as cancer via the RNA interference (RNAi) mechanism. Since the systemic administration of siRNAs is limited by their capacity to attain the site of action, novel delivery systems are needed. Previously, we reported the formulation of magnetic siRNA nanovectors (MSN) using electrostatic assembly of the following components: (1) functionalized superparamagnetic iron oxide nanoparticles (SPIONs) able to act as agents for magnetic resonance imaging (MRI) and/or thermal therapy, (2) siRNAs as active molecules and (3) chitosan to protect siRNAs and to enhance their transfection efficacy.

In this work, experimental design was used to further improve the formulation protocol and to optimize the component quantities. The aim was to obtain response surface plots that will help to optimize and predict the component quantities of the MSNs regarding their hydrodynamic diameter (D_H). The influent parameters of the formulation process were determined using a Plackett–Burman design. The results show that the order of incorporation of the components is the most influent parameter on the D_H of MSNs. A Box–Behnken design was used to optimize the component quantities. The model equations provided the parameters to obtain MSNs with D_H smaller than 100 nm to allow their systemic administration.

1. Introduction

The use of short interfering RNAs (siRNAs) is a promising direction in development of targeted molecular anticancer therapies. SiRNAs are short double stranded RNA molecules composed of 22 pairs of nucleotides. To diminish or inhibit the protein synthesis, the siRNAs have to be integrated in the endogenous RNAi (RNA interference) mechanism. Once in the cellular cytosol, the siRNA duplex is incorporated in the Argonaut complex and the sense (or passenger) strand is degraded. The antisense (or guide) strand is then incorporated in the RNA-induced silencing complex (RISC) which will guide and associate the siRNA simplex to the target mRNA containing the complementary sequence. This action leads to inhibition of the gene expression either via degradation of the mRNA or via inhibition of the translation (Meister and Tuschl, 2004). However, application of siRNAs is limited by their properties, especially if they have to be administered by intravenous injection: (i) they are likely to be rapidly degraded by nucleases in the bloodstream, which reduces their blood half-life, (ii) they have no specific targeting sequence to help them in reaching the genetically modified target cells, (iii) their negative charges hinder crossing similarly charged cellular membranes to attain the cytoplasm (Oh and Park, 2009; Castanotto and Rossi, 2009). To enhance siRNA efficacy and circumvent these barriers, a delivery system should be used. For this, nanocarriers based on superparamagnetic iron oxide nanoparticles (SPIONs) can be of particular interest.

In this aim, magnetic siRNA nanovectors (MSN) composed of SPIONs, chitosan and siRNAs were previously developed. SPIONs are composed of magnetite and maghemite and are therefore highly magnetized in a magnetic field, but lose their magnetization when the field is switched off (so-called superparamagnetic behavior). This behavior is essential for injectable formulations, because it reduces the risk of thrombosis from magnetically

Abbreviations: D_H, hydrodynamic diameter; MSNs, magnetic siRNA nanovectors; RNAi, RNA interference; siRNA, short interfering RNA; SPIONs, superparamagnetic iron oxide nanoparticles; MRI, magnetic resonance imaging; RISC, RNA induced silencing complex; DOE, design of experiments; MR, mass ratio; CR, charge ratio; IS, ionic strength; F01, first formulation protocol ((chitosan + SPIONs) + siRNA); F02, second formulation protocol ((siRNA + SPIONs) + chitosan); F03, third formulation protocol ((siRNA + chitosan) + SPIONs); PDI, polydispersity index.

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aggregated NPs. Furthermore, the magnetic properties of SPIONs allow their use as (i) imaging agents in MRI, and (ii) agents for thermal therapy which consists in local heating by alternating magnetic fields, leading to accelerated drug release or even to destruction of cancer cells (hyperthermia therapy). Chitosan is a natural polysaccharide composed of glucosamine and *N*-acetylglucosamine. For almost twenty years, it has been investigated and developed for nucleic acid delivery. The chitosan layer is thought to protect siRNA molecules from degradation and can act as transfection agent for nucleic acids (Buschmann et al., 2013; Malmo et al., 2012; Rudzinski and Aminabhavi, 2010; Wang et al., 2011). The nanocarriers described here were obtained using self-assembly via electrostatic interactions. For this purpose, the SPION surface was beforehand modified with amino-silanes that carry positive charges at physiological pH. SiRNAs are negatively charged due to their phosphodiester backbone, and the chitosan is rather positively charged at the formulation pH of 5.5 (pKa = 6.5) (Buschmann et al., 2013). The association of the three components results in the formation of MSNs. The feasibility of the MSN formulation and characterization have been validated recently (David et al., 2013). Combination of the above mentioned therapeutic and diagnostic functions, as in the case of MSNs, is called theranosis.

The size of nanovectors is a parameter of the formulation that is essential to take into account when an intravenous administration is envisaged. It is generally admitted that the nanovector's size should not exceed 100 nm to reduce the recognition, activation and elimination by the immune system (Vonarbourg et al., 2006a). However, their size should be bigger than 20 nm to prevent natural renal clearance (Choi et al., 2007). As the silanised SPIONs used to formulate MSN, are already around 40 nm, we will limit us to a minimum D_H of 50 nm. Thus, our aim is to formulate MSN with a hydrodynamic diameter (D_H) between 50 and 100 nm. In the literature, the D_H of chitosan-siRNA nanoparticles, varies between a few tens and a few hundreds of nanometers depending on the preparation method (ionic gelation, self-assembly, etc.), the protocol (concentration of the constituents, pH, volumes mixed etc.), the properties of chitosan (MW, degree of deacetylation, origin) and the suspension medium (Buschmann et al., 2013; Malmo et al., 2012; Rudzinski and Aminabhavi, 2010). To identify the relationship between these factors and the size of chitosan nanoparticles, experimental design methodology can be used (Elmizadeh et al., 2013; Patel et al., 2013).

Keeping this in mind, experimental design methodology was applied to understand the influence of the different formulation parameters on the D_H of MSNs and to optimize the formulation protocol. The aim of using this methodology is twofold: (i) to estimate and compare the effects of the parameters that affect the system (here the D_H of the MSN formulation) and (ii) to model precisely the behavior of the system which will help to predict the D_H of MSNs. In the previous study (David et al., 2013), a linear model in form of a full factorial 2^4 design was used to analyze the parameters influencing the D_H of the formulations. However, the statistical analysis of this model showed a significant lack of fit indicating a curvature of the response surface not taken into account by this linear model. For this reason, we used this time a quadratic model in the form of a Box–Behnken design which can model the behavior more precisely.

During the first step of this work, experimental design methodology in the form of a Plackett–Burman design was employed on three different formulation protocols. The Plackett–Burman design is a screening design that can be used to identify the parameters that have an influence on the selected response. In this study, the parameters are the different operating conditions and the response is the size of MSNs. One advantage of using the Plackett–Burman design is that the influence of seven parameters can be analyzed simultaneously with only eight experiments. The drawback is that only the main effects can be determined and neither interactions nor surface response modelling can be established (Hibbert, 2012).

The second step was to analyze the influence of the component quantities on the D_H of MSNs. Therefore, the Box–Behnken design was chosen, allowing to identify the main effects, the two-way-interactions and the quadratic main effects as well as to draw a response surface plot to optimize the parameters (Hibbert, 2012; Ferreira et al., 2007). This design was applied using the two formulation protocols that were selected regarding the results of the Plackett–Burman experiments. Four experimental parameters were varied simultaneously in order to analyze the parameters influencing the D_H. In parallel, response surface plots were created, helping to predict the parameter ranges necessary to obtain the chosen D_H.

2. Materials and methods

2.1. Nanocarrier preparation

Magnetic siRNA nanovectors (MSN) were composed of siRNA (here a model siRNA targeted against PCSK9, Eurogentec, Seraing, Belgium), silanised SPIONs (Hervé et al., 2008), chitosan (chitosan high purity, MW 110,000–150,000, Sigma–Aldrich Chemie GmbH, Schnelldorf, Germany), $NaNO_3$ (sodium nitrate extra pure, DAB6, Merck, Darmstadt, Germany) and water (obtained from a Milli-Q system, Millipore, Paris, France) as described in Ref. (David et al., 2013).

Briefly, MSNs were prepared by mixing equal volumes of two aqueous preparations using a micropipette according to three different formulation protocols (F01, F02 and F03) (Fig. 1). The difference in the three formulation protocols was the order in which the different components, siRNA, SPIONs, and chitosan were added. In the first protocol, the one already published (David et al., 2013), an aqueous suspension of SPIONs and an aqueous solution of chitosan are mixed together before the addition of an aqueous solution of siRNAs. In the second protocol, an aqueous solution of siRNAs was first mixed with an aqueous suspension of SPIONs (F02) and in the third protocol we start out with a mixture of siRNA and chitosan (F03). The composition of the MSN formulation was determined by four parameters: (i) the mass ratio of silanised SPIONs (defined as iron content) to siRNA (MR = m(Fe)/m(siRNA)), (ii) the charge ratio (CR) of positive chitosan charges (amine groups) to negative siRNA charges (phosphate groups), (iii) the ionic strength (IS, determined by the sodium nitrate ($NaNO_3$) concentration) and (iv) the siRNA concentration ([siRNA]). The ionic strength was fixed by adding $NaNO_3$, rather than sodium chloride, to avoid interactions of iron-based SPIONs with chloride ions. For the Plackett–Burman experiments, the parameters were fixed at MR = 20, CR = 65, IS = 0.05 M and siRNA concentration = 30 μg/ml. For these experiments $NaNO_3$ was added, either in the first or in the second tube depending on the protocol and the experiment (Table S1, Supplementary Data). For the Box–Behnken experiments, the parameters varied in function of the experiments (Table S3, Supplementary Data) whereas $NaNO_3$ was added in the second tube in each case.

2.2. Nanocarrier characterization

2.2.1. Size determination

The size of the nanocarriers was measured in terms of their mean hydrodynamic diameter (D_H) using a high performance particle sizer (HPPS) (Malvern Instruments, Malvern, UK). Before measurement, the nanocarriers were diluted in $NaNO_3$ 0.01 M at a ratio of 1:25. The D_H measurements of the nanocarriers were done