LARGE SIZE DNA *IN VITRO* AND *IN VIVO* DELIVERY USING CHITOSAN TRANSFECTION

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Abstract

In modern medicine, many diseases can be treated using gene therapy, which requires a DNA delivery system to prevent DNA from degradation and to transport it to the cells. Liposomal reagents are expensive for such a therapy and new inexpensive biodegradable DNA carriers are required. Chitosan (Ch) is a cationic polymer with promising potency for gene delivery. Some Ch derivatives have been shown to efficiently transfect mammalian cells in vitro. However, there are many inconsistencies in the literature concerning the effectiveness of Ch systems for in vivo gene transfer. The aim of this work was to develop a Ch-based vector for in vivo delivery of a large-size DNA fragment coding for the far-red fluorescent protein (RFP). Among several Ch derivatives, hexanoyl-Ch (HCh) with a molecular weight of 20 kDa effectively transfected cells in vitro. Intratumoural injection of polyplexes in colon and lung tumours resulted in local expression of RFP in tumours.

Key words: *chitosan derivatives, DNA delivery, hydrophobic groups, pRFP pmKate2 reporter plasmid*

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1. Introduction

Transfection efficacy of chitosan (Ch) with plasmid DNA (polyplexes) primarily depends on the properties of Ch, such as its molecular weight (MW) and degree of deacetylation (DD). It was shown that polyplexes of low (7–18 kDa) to medium (40–60 kDa) MW Ch were more effective in comparison with high MW Ch (>150 kDa) [1–4]. Good results were shown after the introduction of hydrophobic substituents, such as hexanoic, octanoic, decanoic [5], dodecanoic [6], stearic [7, 8] or linoleic [9] short-chain fatty acids. Analysis of *in vivo* gene transfer requires a robust detection system. Reporter genes such as GFP allow *in vitro* flow cytometry and confocal microscopy quantitative assessments; however, they are not good for *in vivo* tracking due to auto fluorescence in this light range. As a result, multiple groups have estimated the *in vivo* efficacy of Ch-based delivery systems using siRNA transfer [2, 10, 11]. However, it is likely that not only the MW of Ch but also the size of DNA plays a role in the resultant transfection efficacy. The MW of GFP, luciferase, and galactosidase are around 310, 560–1120 and 930 kDa, respectively, while siRNA MW is only 8 kDa. This difference can significantly affect polyplex formation. Oliveira *et al.* [12] tried to develop a universal Ch-based vector for the delivery of small and large plasmids; however, this work resulted in a much more sophisticated system. In previous work, we identified that hexanoyl-chitosan with a MW of 20 kDa efficiently transfected human embryonic kidney cells (HEK293) [13]. The aim of the present work was to develop a Ch-based vector for the *in vivo* delivery of a large size DNA fragment coding for far-red fluorescent protein (RFP). Here, we synthesised hexanoyl-chitosan derivatives with different MWs (5, 20, and 50 kDa) and different substitution degrees (SD) and studied their efficacy as *in vitro* and *in vivo* DNA transfer reagents for far-red fluorescent protein coded by the reporter plasmid pRFP pmKate2 [14].

2. Materials and Methods

2.1. Materials

Chitosan with MW 1000 kDa and DD 90% was purchased from ZAO "Bioprogress", Russia. Glacial acetic acid, sodium hydroxide, hydrochloric acid, acetic and hexanoic anhydrides and methanol (Sigma-Aldrich, USA) were used as purchased.

2.2. Synthesis

Low MW Ch (Samples 4–12, Table 1) was obtained from Ch 1000 kDa by acidic hydrolysis as previously described [15]. Hydrophobic derivatives were synthesised using anhydrides of hexanoic acid. The DD and SD of Ch derivatives were determined by ^{1}H-NMR.

2.3. Cells

HEK293 were grown in DMEM supplemented with 10% foetal calf serum (FCS) and pen-strep-glut (all from PanEco, Moscow, Russian Federation). Cells were passaged using Trypsin/EDTA solution (PanEco, Moscow, Russian Federation) twice a week. Twenty-four hours before the assays, cells were seeded in the appropriate plates (6- or 24-well plates) adjusted to $3x10^5$ cells/mL and incubated overnight to achieve standardised growth conditions.

2.4. Transfection

Bright far-red fluorescent protein coded by the reporter plasmid pRFP pmKate2 [13] (Eurogene, Moscow, Russian Federation), MW 1656 kDa, was used to analyse Ch transfection efficacy. Ch samples were dissolved in 0.1% acetic acid at 5 mg/mL. To

form polyplexes, 10 μL of Ch derivatives were mixed with 2–3 μg of plasmid in 50 μL of serum-free medium and incubated for 20 min. A commercial liposomal transfection reagent Metafectene Pro (MF) (Biontex, Germany) was used as a control. To prepare the polyplex, 3 μL Metafectene Pro was dissolved in 50 μL serum-free medium and mixed with 1 μg of pRFP dissolved in 50 μL. Polyplexes were formed over 20 min and added drop-wise to cells.

2.5. Confocal analysis

For confocal analysis, cells were grown overnight on sterile cover slips in 200 μL of complete culture medium in 6-well plates (Costar). Warm serum- and antibiotic-free 1 mL medium was added to polyplexes and the mixture was added to the wells. Cells were incubated for 1 h and then 2 mL of complete medium was added. Before the analysis, cells were fixed with 1% paraformaldehyde, washed, and polymerised with Mowiol 4.88 medium (Calbiochem, Germany). Slides were analysed using an Eclipse TE2000 confocal microscope (Nikon, Japan).

2.6. Flow cytometry

The total level of transfection was estimated by flow cytometry. For this, HEK293 cells were seeded in 24-well plates overnight, transfected as described above, and incubated for 72 h. Before the analysis, cells were trypsinised, washed in pre-warmed complete culture medium, and analysed using a FACScan device (BD, USA).

2.7. Dynamic light scattering

The diameters of polyplexes were characterised by dynamic light scattering (DLS) (90 Plus Particle Size Analyzer Brookhaven Instruments Corporation, Vernon Hills, IL, USA). All measurements were performed using a 661 nm laser light at room temperature with a 90° angle of detection. The zeta potential of Ch was determined in 10 mM KCl using identical equipment with an additional ZetaPALS apparatus. Measurements were performed at 25.0 ± 0.1°C.

2.8. Tumour models

BALB/c and C57BL/6 mice were purchased from Pushchino Farm, Moscow District, at the age of 3 months. Mice were kept under minimal disease conditions. All experiments were conducted according to the Institutional Animal Commission protocol. BALB/c mice were inoculated s.c. in the right flank with 10E6 CT26 cells (murine colorectal cancer [16]). C57BL/6 mice were inoculated with LLC (Lewis lung carcinoma [17]). Tumours were grown until 1–2 mm^3 in volume. Polyplexes were injected in the distal part of the tumours, the needle was kept parallel to the skin to avoid injection in the central blood vessel. Mice were sacrificed 48 h after transfection. Tumour, liver, and spleen were collected, fixed in paraforaldehyde for 24 h, soaked in 30% sucrose overnight, and cryosectioned using a ThermoScientific FSE Cryotome (USA).

3. Results and Discussion

3.1. Selection of Ch derivative for in vitro transfection

Physicochemical characteristics of Ch derivatives are shown in Table 1.

Representative ^{1}NMR spectra for samples **4–6** are presented in Fig. 1. The diameters of polyplexes formed by both MF and HCh were around 250–400 nm, as determined by DLS

Table 1. Characteristics of hydrophobic chitosan derivatives

#	**Derivatives**	**MW**	**DD**	**SD**	**ζ^a, mV**
1–3	[e]HCh50	50	90	8/13/27	+(32–35)
4–6	HCh20	20	90	6/13/21	+(20–27)
7–9	HCh5	5	90	4/12/18	+(20–25)

MW – molecular weight, kDa. DD – deacetylation degree, %., SD – substitution degree for carbonic acid groups, %, [a]ζ – zeta-potential, mV.
[e]HCh – Hexanoyl chitosan with 50, 20 or 5 kDa MW.

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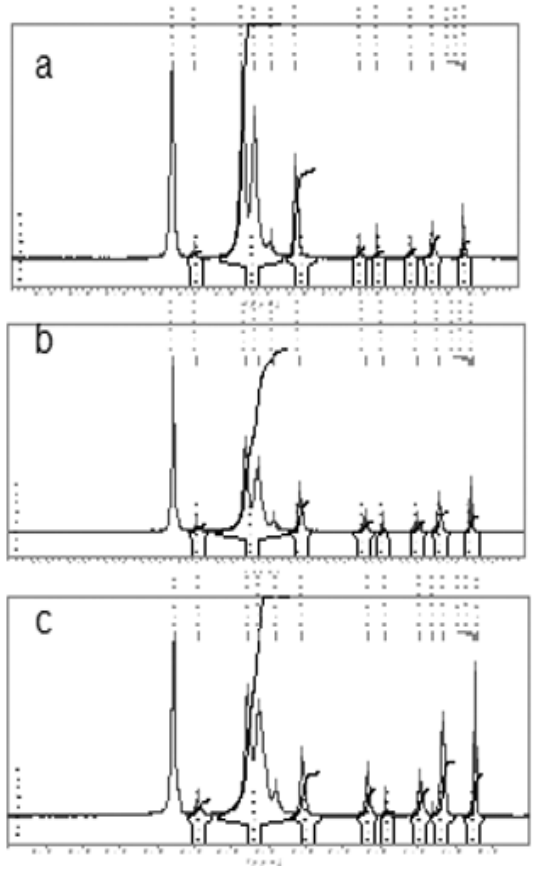

Figure 1. [1]NMR spectra of Ch derivatives. HCh20/6 (a), HCh20/13 (b), HCh20/21 (c).

Transfection of HEK293 was conducted using a commercial lipid reagent, MF, or HCh samples under identical conditions. Low MW samples were not able to pack the plasmid and transfect the cells (data not shown). HCh50 had a lower efficacy than HCh20 (Fig. 2). In the experiment presented in Fig. 2, the best transfection was found for HCh20 with low SD of 6% (Fig. 2, b, h). The results of four experiments demonstrated that all HCh20 samples effectively transfected HEK293 cells; however, the efficacy slightly varied in different experiments. It was concluded that MW, not SD,

is the most important parameter. On average, the transfection efficacy of HCh20 was 1.5–2 times lower than of the lipid reagent MF (Fig. 2, a. e). Of note, the unmodified Ch20 was unable to transfect cells, showing that some hydrophobic groups are essential for DNA condensation. This result demonstrates that the combination of 1656 kDa DNA and HCh20 with any SD can be used for *in vivo* transfection.

3.2. In vivo efficacy Ch derivatives

Cancer is one of the most important applications of gene therapy. We used two types of subcutaneously transplanted tumours, modelling colon and lung cancer. Subcutaneously transplanted tumours in mice form a central blood cavity (Fig. 3). Injection of polyplexes in the tumour centre will inevitably result in the distribution of the injected material throughout the body. Thus, we tried to inject polyplexes parallel to the skin surface (Fig. 3, red arrows). To mark the place of injection, fur was removed locally by an epilator and the place of injection was marked. A total of 4 μg of RFP plasmid was injected. Mice were sacrificed 48 h later, and the place of injection in the tumour was excised, fixed, cryosectioned and analysed by confocal microscopy. Representative results for control, MF and HCh20/13 are shown in Fig. 4. All three samples of HCh20 effectively transfected both types of tumour cells.

In addition to tumour injections, the same amount of polyplexes was injected intramuscularly (i.m.) in the thigh opposite to the tumour. I.m. injection was less efficient in comparison with tumours; however, all polyplexes delivered the RFP plasmid to the muscular cells (Fig. 5, a–g).

Finally, it was interesting to estimate whether polyplexes were delivered centrally after intratumoural injection. This experiment was conducted in LLC tumour bearing mice. It appeared that polyplexes were delivered at least to the spleen and liver (Fig. 5, h–j).

There are many inconsistencies with *in vitro* and *in vivo* results concerning the effectiveness of Ch systems for gene transfer [18]. Ch is an attractive biopolymer with abundant reactive amino groups, which can be used to obtain functional conjugates. However, the flexible Ch structure constrains the regular packaging of DNA. The major focus of Ch gene delivery research is directed to the role of Ch MW, deacetylation degree and different substitution groups. Much less, if any, attention is paid to the size of DNA that should be packed in the Ch system. Reporter genes, such as GFP, luciferase and galactosidase, are often used [19]. Multiple groups have therefore developed Ch-based delivery systems for siRNA transfer [2].

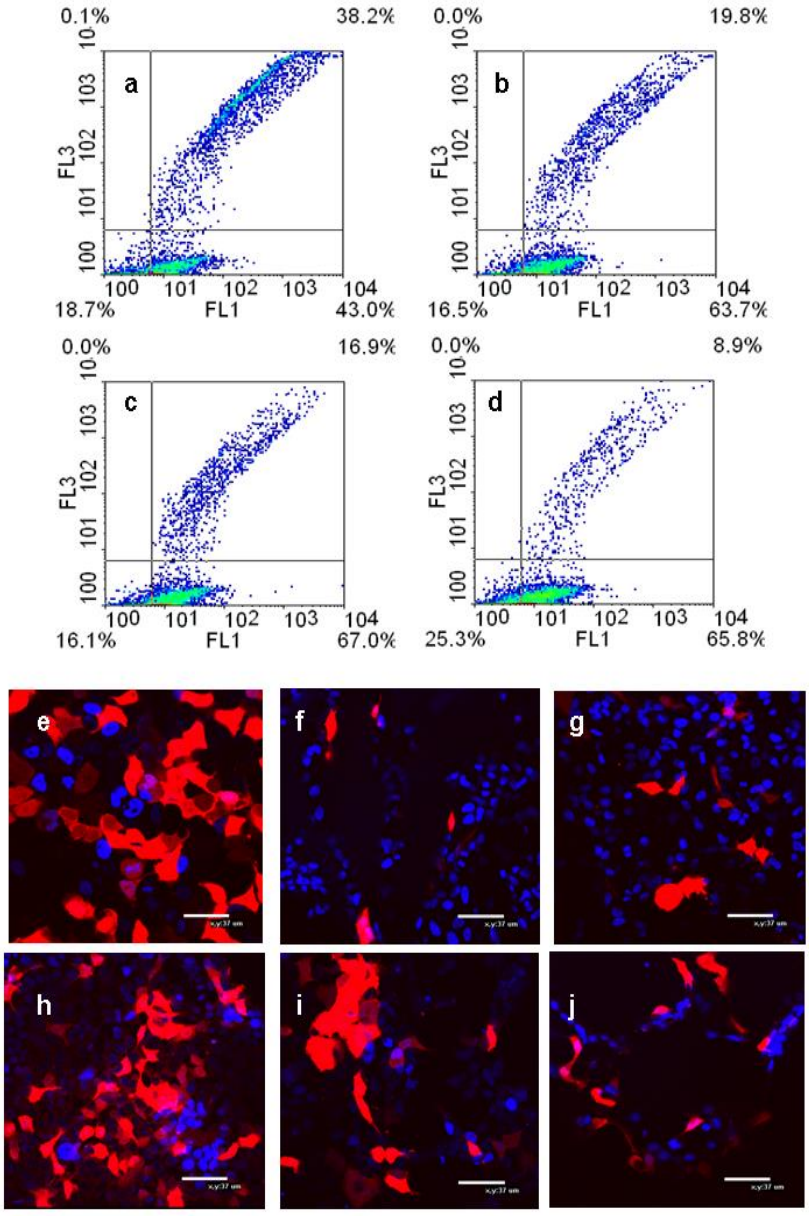

Figure 2. Transfection efficacy of Ch derivatives of HEK293 cells with RFP *in vitro*. a–d. Flow cytometry analysis of transfection efficacy of MF (a), HCh20/10 (b), HCh20/15 (c), and HCh20/30 (d). Confocal images of HEK293 cells 48 h after transfection using MF (e), HCh50/13 (f), HCh50/27 (g), HCh20/6 (h), HCh20/13 (i), and HCh20/21 (j). The red colour shows transfected cells, blue colour shows nuclei stained with Hoechst. Scale bar corresponds to 40 μm.

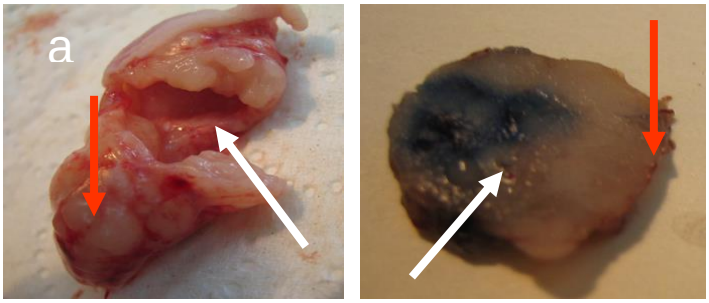

Figure 3. Formation of blood cavity in transplanted tumours. Both CT26 (a) and LLC (b) form large blood cavity (shown by white arrows). A mouse with LLC tumour was injected i.v. with Evans blue dye, which marks blood deposition (b, white arrow). Injections were made parallel to skin (shown by red arrows).

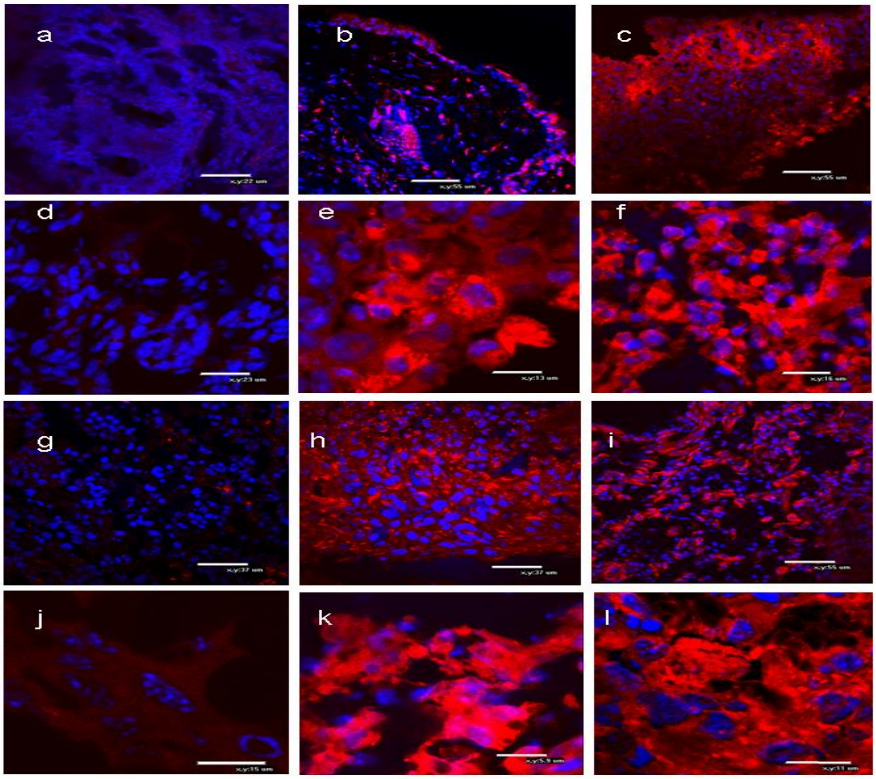

Figure 4. Confocal images of *in vivo* transfection of tumours. HT26 (a–f) or LLC (g–l) implanted tumours were transfected using intratumoural injection of polyplexes RFP with MF (b, e, h, k) or HCh20/13 (c, f, I, l). Control tumours (a, d, g, j). Red colour corresponds to RFP expression, nuclei are stained with Hoecst 33342 (blue). Scale bar corresponds 220 μm (a–c, g–i) or 40 μm (d–f, j–l).

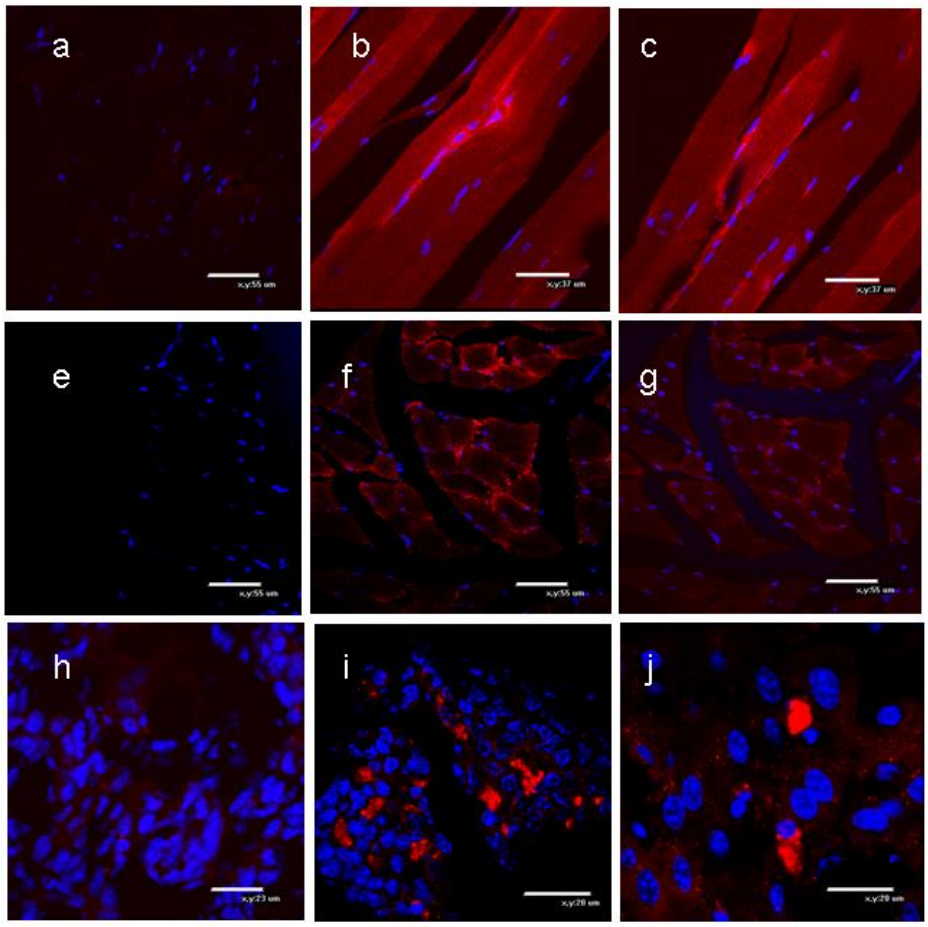

Figure 5. Confocal images of cryosections after intramascular *in vivo* transfection. a–g. Mice were inoculated i.m. with polyplexes formed by RFP and MF (b, f) or HCh20/13 (c, g). Control muscles (a, e). Longitudinal (a–c) and cross (e–g) sections are shown at 48 h post injection. Scale bars correspond to 50 μm. (h–j). MF-RFP polyplexes were injected into LLC tumours. Spleen (h, i) and liver (j) were collected 48 h post injection. The red colour corresponds to RFP expression, nuclei are stained with Hoecst 33342 (blue). Scale bars correspond to 20 μm.

The MW of siRNA, GFP, luciferase, and galactosidase are around 8, 310, 560–1120 and 930 kDa, respectively. This difference can significantly affect polyplex formation and can explain the variability in the results obtained. Oliveira *et al.* [12] tried to develop a universal Ch-based vector for the delivery of small and large plasmids; however, it resulted in a much more sophisticated system. In this work, we used a new reporter plasmid pRFP pmKate2 of MW 1656 kDa, which encodes far-red fluorescent protein. Far-red fluorescent proteins are good candidates to estimate the *in vivo* fate of the plasmid as the emission of GFP and luciferase falls in the range of autofluorescence, while the galactosidase reporter requires dye extraction. Moreover, a delivery system for

large size DNA can be used to carry gene-coding functional proteins. We have demonstrated that a combination of a 1656 kDa plasmid and HCh20 with SD 4–27% works well both *in vitro* in HEK293 cells and *in vivo* in colon and lung carcinomas. Variations in MW from 20 to 50 kDa and 5 kDa resulted in the loss of transfection activity. As some level of transfection was found in HCh50 polyplexes, there is a possibility that the activity will be preserved in the range of 20–40 kDa. It is interesting that close results on the efficacy of hydrophobic Ch derivatives were obtained by Layek and Singh [5], who used pGFP and 50 kDa Ch derivatives to form polyplexes. They also obtained the best results with hexanoyl derivatives in comparison with N-octanoyl and N-decanoyl derivatives, while all samples effectively transfected the cells *in vitro* [5]. The results of Layek and Singh also support our hypothesis on the major role of Ch MW and a lesser role for the hydrophobic moiety type and SD. The size of pGFP is around 310 kDa. It would be interesting to compare the efficacy of HCh20 to deliver pGFP.

I.m. injection is another route for gene therapy. We demonstrated that HCh20 also delivered pRFP to muscular cells. Earlier i.m. injection of NKG2D-IL-15 fusion gene plexed with N-(2-hydroxyl) propyl-3-trimethyl ammonium Ch resulted in the suppression of tumour growth [20]. Earlier, we attempted to use quaternised Ch and quaternised HCh20 to deliver pRFP; however, both derivatives failed to transfect cells *in vitro* [13].

Finally, we showed that during intratumoural injection, polyplexes were released into the blood. This is more likely to be true for the transplantable mouse models of tumour growth: these tumours form a large blood depot inside. This means that intratumoural therapy in such models should be conducted with care.

4. Conclusions

Chitosan, a biodegradable renewable inexpensive cationic polymer with abundant amino groups, is ideally suited to developing multifunctional conjugates and gene delivery systems of therapeutic potential. However, there are many inconsistencies in the literature concerning the effectiveness of different chitosan derivatives for both *in vitro* and *in vivo* gene transfer. This difference can be the result of poor understanding that both the characteristics of chitosan and the size of DNA affect DNA condensation and the resultant transfection efficacy. Moreover, even chitosan MW is not determined completely. In previous work, we have shown that chitosan MW determined by different methods results in 2–10 times discrepancy [21]. Here, we demonstrated that among nine N-hexanoyl-chitosan derivatives with different MW and SD, only three demonstrated a superior transfection rate both *in vitro* and *in vivo* when plexed with a plasmid pRFP pmKate2 coding for far-red protein RFP. N-hexanoyl-chitosan with MW 20 kDa and SD of 6–21% demonstrated equal efficacy both *in vitro* in HEK293 cells and *in vivo* in colon and lung carcinoma tumour models in mice. A decrease in MW to 5 kDa completely abolished transfection activity, while an increase in MW to 50 kDa decreased it significantly. At the same time, the number of hexanoic groups in HCh20 had very little effect on the transfection rate. Thus, a narrow window of chitosan and DNA MW should be found for each gene of interest.

5. Acknowledgements

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There are no financial or other issues that might lead to conflict of interest.

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