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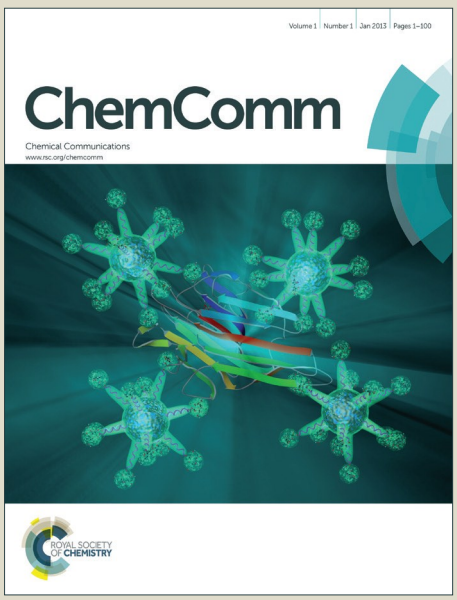

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Construction of negatively charged and environment-sensitive nanomedicine for tumor-targeted efficient siRNA delivery

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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A novel siRNA delivery system based on triblock copolymer with pH and reduction dual-sensitivity was introduced. The polyplex has high delivery efficiency not depending on surface charge reversion in response to the pH value of tumor tissue, was used for target gene silencing in cancer therapy.

RNA interference (RNAi) provides a great potential for cancer therapy due to its highly efficient target gene silencing.[1,2] However, its clinical applications has been greatly impeded by the issues of siRNA delivery.[3,4] Especially, the siRNA molecules are easily degraded by nuclease before reaching the target sites *in vivo*. In addition, the negatively charged siRNA molecules are unable to cross cell membrane and other biological barriers, which also make their clinical applications impossible. Thus, it is crucial to develop an effective delivery system when seeking siRNA-mediated gene silencing for cancer therapy.

Cationic polymeric vectors for siRNA delivery have drawn great attention in recent years due to their less immunogenicity, less toxicity and easily tailorable structures meeting therapeutic needs.[5,6] Up to date, numerous cationic polymers have been investigated as siRNA carriers, such as poly(ethylenimine) (PEI),[7] poly(L-lysine),[8] imidazole-containing polymers,[9] chitosan,[10] and cationic dendrimers.[11] Through electrostatic interaction, the anionic siRNA molecules may complex with cationic polymers to form nano-sized polyplexes which can accumulate in tumor *via* an enhanced permeability and retention (EPR) effect.[12] To well protect siRNA from enzymatic degradation *in vivo* and to enable cell uptake by endocytosis, the polyplexes are usually formed at relatively high N/P ratios and thus are positively charged. Unfortunately, the application of the positive polyplexes *in vivo* is limited by their non-specific cell interaction, short blood circulation, high cationic toxicity, and aggregation induced by protein adsorption in bloodstream.[4,13] On the other hand, polyplexes formed at low N/P ratios are negative-charged, which endows the polyplexes with better biocompatibility and potentially long circulation in bloodstream. However, their cell uptake is difficult due to the poor affinity to cell membrane which is likewise negatively charged. Furthermore, siRNA is not fully complexed in polyplexes formed at low N/P ratios and thus is still subject to enzymatic degradation *in vivo*.[14] Therefore, development of polyplexes with minimal nonspecific cell interactions and meanwhile combing the advantages of the two types of polyplexes, e.g. long blood circulation, efficient cell uptake, sufficient siRNA protection and low cytotoxicity, is still of great importance for cancer gene therapy nowadays.

A strategy using surface charge conversion has been commonly adopted to achieve the goal, which mainly depended on the strong proton-buffering capacity of PEI[15] and the lower pH value (≈ 6.8) of tumor extracellular space caused by deficient metabolism of lactic acid.[16] Given a precise control of the N/P ratio in preparation, the polyplex could be negatively charged at neutral pH of bloodstream but positively charged at lower pH of tumor tissue.[17] In particular, polyplex with such specific pH response was successfully obtained by complexing siRNA/pDNA with branched PEI ($bPEI_{25K}$) and a copolymer poly(PEG-His-PEG-Glu).[17,18] Despite the great potential in improving delivery efficiency of nucleic acids *in vivo*, the approach relied much on a harsh control of the ratio of $bPEI_{25K}$/nucleic acid/poly(PEG-His-PEG-Glu). This particular ratio at which polyplex could show opposite surface charges at pH 7.4 and 6.8 was hard to locate, because the two pH's are too close and even slight molecular changes of PEI or copolymer may lead to alteration of the required ratio. In addition, even though the acute cytotoxicity of vector was lowered by the introduction of negatively charged poly(amino acid)s, the *in vivo* accumulative toxicity of $bPEI_{25k}$ due to its nondegradability was still a concern for long term therapy. Using a different approach of reduction-sensitive interlayer

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Electronic Supplementary Information (ESI) available: Materials, detailed experimental procedures and characterization data. See DOI: 10.1039/x0xx00000x

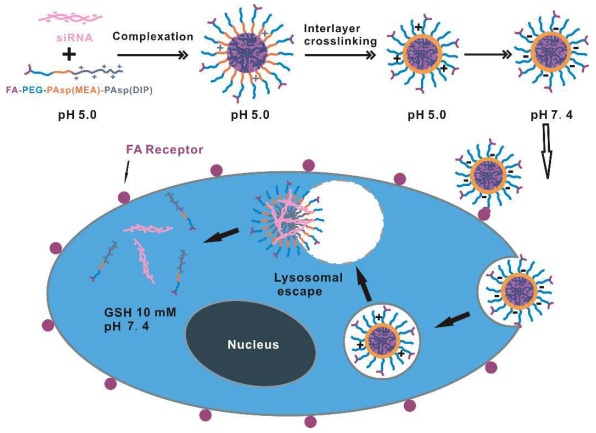

Fig. 1 Illustrative preparation and intracellular fate of negatively charged and folate-targeted polyplex with reduction and pH dual sensitivity.

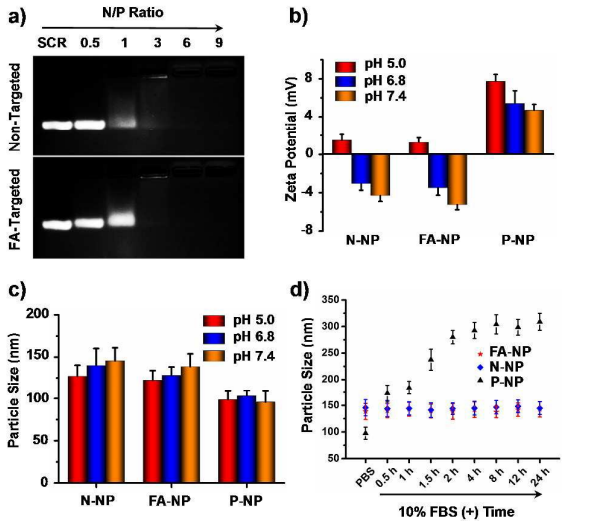

Fig. 2 a) Electrophoretic mobility of SCR (Scrambled siRNA) in agarose gel after complexing with non-targeted and FA-targeted polymer at various N/P ratios in PBS 5.0. Zeta potentials b) and particle sizes c) of polyplexes at different pH values. d) Stability of polyplexes in PBS (pH 7.4) containing 10% fetal bovine serum (FBS) measured by dynamic light scattering (DLS). Data are mean ± SD in sub-figures b, c and d (n = 5).

package, we recently prepared another surface charge-reversible polyplex based on low molecular weight lPEI with low accumulative toxicity.[19] Owing to the interlayer crosslinking and pH-induced surface charge conversion, the polyplex may circulate longer in bloodstream, well protect siRNA against nuclease degradation, and effectively enter the tumor cells inside tumor tissue. Still, the approach needs careful control of a particular N/P ratio of PEI to siRNA in order to achieve opposite surface charges at the two close pH values, i.e. pH 7.4 and 6.8.

The present study aimed to develop an easy to control vector with high siRNA delivery efficiency not depending on a surface charge reversion of polyplex caused by pH change in the narrow range between 7.4 and 6.8. To this end, we propose to prepare the siRNA nanomedicine based on a combined strategy utilizing active tumor targeting, polyplex interlayer crosslinking and carrier proton-buffering (Fig. 1). The polymeric vector designed for the implementation of our strategy is a folate-terminated biodegradable triblock, FA-PEG-PAsp(MEA)-PAsp(DIP), of polyethylene glycol (PEG), poly(aspartic acid-cysteamine) (PAsp(MEA)) and poly(aspartic acid-diisopropylethylenediamine) (PAsp(DIP)) (Scheme S1 and S2, ESI). Compared to our recent work, [20] the new features of the polymer described herein include: 1) The polymer was tailor-made for siRNA delivery. A much longer PAsp(DIP) block was designed for well siRNA complexation and meanwhile a much shorter PAsp(MEA) middle block was introduced for reduction-triggered siRNA release; 2) Synthetic approach was modified for low risk of vector toxicity, as amidation reaction rather than click chemistry introducing toxic copper ion difficult to eliminate was adopted to couple different blocks; 3) Active targeting is introduced, which was demonstrated a key for effective siRNA delivery. In addition, a distinct feature of the above vector is that PAsp(DIP) based on the biodegradable polypeptide rather than the well-known PEI was employed as a proton-sponge for lysosomal escape of polyplex and intracellular release of siRNA. The successful synthesis of polymers *via* multi-step reactions was confirmed by ^{1}H NMR, FTIR and GPC analyses (Fig. S1-S8, ESI). Since more DIP groups of the PAsp(DIP) block are protonated for siRNA complexation at acidic condition, pH 5.0 rather than pH 7.4 was chosen to form the polyplexes. Complexation of polymer with siRNA at pH 5.0 prior to interlayer crosslinking was evaluated by agarose gel electrophoresis. When the N/P ratio increased, stripes of free siRNA became weak and finally disappeared around the N/P ratio of 6, indicating a just full complexation of siRNA (Fig. 2a). Moreover, conjugation of folate to the copolymer seemed to have no appreciable effect on siRNA complexation. Since a positive surface charge inside lysosomes (≈ pH 5.0) and a negative surface charge in bloodstream (pH 7.4) are favorable for the lysosomal escape and long circulation/low cytotoxicity of polyplexes respectively, N/P 6 was selected to prepare polyplexes for further studies. After interlayer crosslinking, the disulfide linkage in polyplex was verified by raman spectroscopy (509 nm, Fig. S9, ESI), and the degree of thiol-to-disulfide conversion was 86.4% according to the measurement of sulfhydryl content.[20]

The non-targeted and folate-targeted nanoscale polyplexes with interlayer crosslinking were denoted as N-NP and FA-NP, respectively. Dynamic light scattering (DLS) measurements showed that N-NP and FA-NP possessed weak positive surface charges at pH 5.0 (Fig. 2b), e.g. +1.48 mV for N-NP and +1.26 mV for FA-NP at pH 5.0, indicating excessive amount of protonated DIP groups over siRNA phosphate groups in these conditions. However, due to the deprotonation of enough DIP groups in the PAsp(DIP) block, both polyplexes became negatively charged at pH 7.4 and 6.8, i.e. -4.29 mV for N-NP and -5.24 mV for FA-NP. Moreover, along with the increase of solution pH from 5.0 to 7.4, no obvious increase in size of the polyplexes was observed ($P > 0.05$). The sizes of N-NP and FA-NP were 145.6 nm and 138.6 nm at pH 5.0 and 127.1 nm and 122.4 nm at pH 7.4, respectively, as measured by DLS (Fig. 2c). These results implied that the interlayer crosslinking

via disulfide bonds might have limited the polyplex expansion driven by insufficient siRNA complexation due to deprotonation of more DIP groups at pH 7.4 (Table S1, ESI). The appropriate sizes and negative surface charges are assumed to assist long circulation of the polyplexes in bloodstream according to our recent study.[19] The polyplex formed at N/P ratio 15 showed surface charges of +4.69 mV at pH 7.4 and +7.72 mV at pH 5.0 respectively, indicating excessive amount of the protonated DIP groups over the phosphate groups of siRNA at both pH's. This polyplex, denoted as P-NP, was used as positive control in the study. Owing to the sufficient siRNA complexation, P-NP showed fairly constant size around 100 nm regardless of pH change between 5.0 and 7.4. In consideration that the size of an anticancer nanomedicine significantly affect its pharmacokinetics and ability to accumulate in tumor tissue through EPR effect,[19] we investigated whether the polyplexes maintain size stability in the presence of blood serum. As shown in Fig. 2d, the negatively charged polyplexes (FA-NP and N-NP) maintained their initial sizes in the pH 7.4 buffer containing 10% fetal bovine serum (FBS) over the experimental time. In contrast, the positively charged polyplex P-NP showed clear size increase against experimental time at the same condition. The above results indicate that, although PEG is well-known to reduce protein adsorption onto nanoparticles, the negative surface charge may also contribute significantly to the antibiofouling ability of nanomedicines,[19,21] which is importantly for their *in vivo* application.

In the TEM observation, FA-NP exhibited uniform size distribution around 130 nm in PBS of pH 7.4 (Fig. 3a). In addition, the polyplex remained intact spherical structure at pH 7.4 plus 5 μM GSH and pH 5 plus 10 mM GSH, indicating good siRNA protection inside bloodstream and lysosomal compartments, respectively (Fig. S10a and b, ESI). However, polyplex disassembly was observed at pH 7.4 plus 10 mM GSH (Fig. S10c), indicating that siRNA release will take place when the polyplex escapes lysosomes and migrates to cytoplasm. The dual-sensitive siRNA release was further demonstrated by fluorometric assay (Fig. 3b and Fig. S11, ESI). At pH 5.0, the fluorescence intensity of FITC was constantly weak against time even in the presence of 10 mM GSH, indicating that FITC-SCR (Scrambled siRNA) was tightly complexed inside polyplex to cause fluorescent quenching. When the solution pH was adjusted to 7.4, only a slight increase of FITC fluorescence intensity was detected, most likely due to the polyplex expansion caused by DIP group deprotonation rather than siRNA release. Moreover, the presence of 5 μM GSH at pH 7.4 did not cause appreciable increase of fluorescence intensity, demonstrating the good stability of polyplex in bloodstream. However, the FITC fluorescence intensity increased significantly against time in the condition of pH 7.4 plus 10 mM GSH (cytoplasm environment), indicating release of FITC-SCR from polyplex which resulted in fluorescence dequenching. These siRNA release data are obviously in line with the TEM results showing structural transitions of polyplex at different conditions (Fig. S10, ESI).

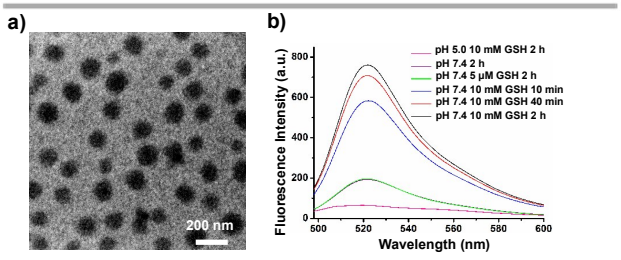

Fig. 3 a) TEM images of FA-NP stained with uranyl acetate at pH 7.4. b) FITC fluorescence intensity changes of FA-NP in solutions of different pH and GSH concentrations. Only in the condition of pH 7.4 plus 10 mM GSH (cytosol-mimicking environment), the FITC fluorescence intensity increased obviously over time, indicating siRNA release. The polyplex FA-NP was formed at N/P=6.

Biological studies were carried out to evaluate the siRNA delivery efficiency of FA-NP in cancer therapy, polyplex of PEI_{25k} complexing siRNA at N/P 6, denoted as PEI-NP, was used as an additional positive control. As determined by MTT assay, compared with PEI and PEI-NP, polymers and three polyplexes at pH 7.4 all showed fairly low cytotoxicity in Bel-7402 cells even at fairly high polymer concentrations up to 0.5 mg/mL (Fig. S12, ESI). To verify folate receptor-assisted cell uptake of FA-NP, Bel-7402 cells were incubated for 8 h with FITC-labeled polyplexes and then visualized under confocal laser scanning microscope (CLSM). As shown in Fig. 4a, unlike the positively charged polyplex (P-NP) which could be taken up by the cells, the negatively charged polyplex without FA targeting (N-NP) could hardly enter cells because of the poor interaction with the likewise negatively charged cell membrane. However, the negatively charged polyplex with FA targeting (FA-NP) was effectively taken up by cells. Moreover, when an excess amount of free FA was added into the cell culture medium, the cell uptake level of FA-NP was dramatically lowered again. Obviously, FA-mediated

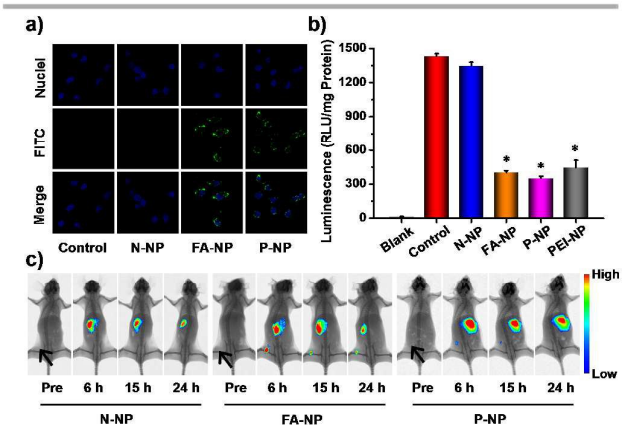

Fig. 4 a) Laser scanning confocal microscopic images (magnification: 630×) of Bel-7402 cells incubated with different polyplexes at pH 7.4 for 8 h. b) *In vitro* luciferase expression in Bel-7402 cells pre-transfected with Luc-pDNA/Lipofectamine2000 and then transfected with different polyplexes carring siRNA targeting luciferase gene at pH 7.4 (siRNA dose: 50 nM). Data are mean ± SD (n = 3, *$P < 0.001$, compared with control and N-NP). c) Typical *in vivo* fluorescence images showing tumor accumulation at different time points after tail vein injection of polyplexes (Dose: 400 μg siRNA/kg body weight; siRNA labeled with AF750). The tumor sites were marked with black arrows.

endocytosis of polyplex occurred *via* specific binding to the folate receptors on cell membranes (Fig. S13, ESI). Determination of quantitative cell transfection efficiency using flow cytometry obtained consistent results. N-NP, FA-NP and P-NP showed cell transfection efficiencies of 0.47%, 64.15%, 75.94%. respectively (Fig. S14, ESI).

In the gene silencing study, we employed the reporter gene luciferase as a model target to evaluate RNA interference efficiency of FA-NP *in vitro*. The Bel-7402 cells were first transfected with luciferase gene using Lipofectamine2000 as a vehicle, and then transfected with the polyplexes carrying siRNA for luciferase. As shown in Fig. 4b, the control group showed a considerably higher level of luciferase expression compared to the blank group without tranfection. The down-regulation of luciferase expression was less than 10% in cells transfected with N-NP. In comparison, much more effective luciferase gene silencing was detected in cells receiving FA-NP. The target gene down-regulation level reached about 70%, equivalent to that induced by the positive P-NP and PEI-NP. These results are in line with flow cytometric results that N-NP could hardly transfect the Bel-7402 cells whereas FA-NP and P-NP could transfect the cells highly effectively. Finally, we investigated the tumor accumulation of polyplexes in nude mice bearing human Bel-7402 hepatoma xenograft. For *in vivo* fluorescence imaging, siRNA was labeled with a near-infrared (NIR) dye AF750, as fluorescence excitation with highly tissue-penetrative NIR light is favorable for clean imaging background.[22] As shown in Fig. 4c and Fig. S15, after tail vein injection, FA-NP accumulated much better in tumor site than both the P-NP and N-NP. Since polyplex with similarly positive charge at pH 7.4 was observed to circulate much short time in bloodstream (Fig. 2d),[19] it appears reasonable to assume that negative surface charge-enabled long circulation and folate-mediated endocytosis of polyplex have synergistically contributed to the effective tumor accumulation of FA-NP. It is also reasonable that the negatively charged polyplex without folate targeting (N-NP) could not accumulate effectively in tumor site, considering that N-NP might not be sequestrated even if having entered there because of the poor cell uptake as already demonstrated (Fig. 4a and Fig. S14, ESI).

In conclusion, a novel easy to manipulate siRNA nanomedicine with high delivery efficiency not depending on surface charge reversion of polyplex was developed based on a tailor-made triblock copolymer FA-PEG-PAsp(MEA)-PAsp(DIP) having pH and reduction dual-sensitivity. Like PEI, the PAsp(DIP) block possesses a proton buffering effect,[20,23] which allowed the polymer to effectively complex siRNA at low pH. The interlayer crosslinking using reducible disulfide bonds simultaneously rendered the polyplex with high stability in bloodstream and capacity of quickly releasing siRNA inside cancer cells. The folate-mediated active tumor targeting enabled easy internalization of the negatively charged polyplex into cancer cells, even if no surface charge convention took place to drive polyplex endocytosis inside tumor tissue. This study provides the first proof of concept demonstration that an effective siRNA nanomedicine with low cytotoxicity and high serum stability may be achieved by using active targeting to direct the delivery of the negatively charged polyplex designed with pH and reduction dual-sensitivity.

This work was supported by National Basic Research Program of China (2015CB755500), the National Natural Science Foundation of China (51225305, U1401242, 51373203 and 21174166) and Natural Science Foundation of the Guangdong Province (2014A030312018, S2012020011070), the Guangdong Innovative and Entrepreneurial Research Team Program (2013S086).

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