Supplementary Material (ESI) for Chemical Communications

Ultra-small water-dispersible fluorescent chitosan nanoparticles: synthesis, characterization and specific targeting

Padmavathy Tallury[a] , Sounitra Kar[a] and Swadeshmukul Santra[a,b,c]* Suwussa Bamrungsap,[d] Yu-Fen Huang,[d] and Weihong Tan[d]

ES1

Materials

Chitosan polymer (low molecular wight), Triton X-100, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride) (EDC) were purchased from Sigma-Aldrich Chemical Co., USA; Fluorescein isothiocyanate (FITC), anhydrous ethanol were purchased from Fisher Scientific. Dialysis cellulose membrane (MWCO, 6-8 kD) was purchased from Spectrum Laboratories (Rancho Dominguez, CA). Deoxyribonucleotides and 5'-carboxyl modifiers were purchased from Glen Research (Sterling, VA). All solvents and reagents were obtained from Fisher Scientific and were used without further purification. CCRF-CEM cells (CCL-119 T-cell, human acute lymphoblastic leukemia) and Ramos cells (CRL-1596, B-cell, human Burkitt's lymphoma) were obtained from American Type Culture Association, USA. All of the cells were grown in RPMI-1640 containing 10% fetal bovine serum (FBS) and 100 IU/mL penicillin-Streptomycin at 37 °C in a humid atmosphere with 5% CO_2. G25 Sephadex size-exclusion column (NAP^{TM}-5) was procured from Amersham Pharmacia Biotech, USA.

Instrumentation

Degree of deacetylation of chitosan was determined by elemental analysis at the Atlantic Microlabs, Norcross, GA. JEOL JEM 1011 100kV transmission electron microscope (TEM) was used to characterize particle size. TEM sample was prepared by placing a drop of the chitosan nanoparticles on a carbon coated copper grid (400 mesh size) followed by air drying. Particle size distribution and zeta potential in deionized water was measured by using the Malvern Zeta Sizer (model: NanoZS) Dynamic Light Scattering (DLS) instrument. The concentration of activated aptamer and the amount of FITC in FITC-Chitosan conjugate polymer was determined

by UV-Vis spectrophotometer (Cary 100, Varian, Inc.,CA). Fluorescence excitation and emission spectra were recorded on SPEX Nanolog (HORIBA Jobin Yvon) spectrofluorometer. Flow cytometric analysis was carried out in FACScan cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Fluorescence imaging was conducted with a confocal microscope setup consisting of an Olympus IX-81 inverted microscope with an Olympus Fluoview 500 confocal scanning system.

Experimental Method

Determination of Degree of Deacetylation (DD)

The degree of deacetylation (DD) was determined by elemental analysis. The elemental composition of the chitosan polymer is Carbon (44.11%), Hydrogen (6.84%) and Nitrogen (7.97 %). Using the following equation (Tian Fet al; Carbohydrate Polymers, 57, 31,2004.)

$$DD = \left(1 - \frac{C/N - 5.14}{1.72} \right) \times 100\%$$

where C/N is the ratio (w/w) of carbon to nitrogen and the degree of deacetylation is determined to be 77.0% .

Viscosity Measurement

Molecular weight of chitosan was determined in triplicates by capillary viscometry at 30 ± 0.05 °C using an Ubbelohde viscometer. Filtered chitosan solutions (0.05-0.2%) in 0.2 M CH_3COOH/0.1M CH_3COONa) were equilibrated to 30 °C prior to measurement of flow times. The intrinsic viscosity (3.56 dL/g) was determined by extrapolation of the graph of reduced viscosity against concentration. The viscosity average M_v was calculated from the intrinsic viscosity [η] using the Mark-Houwink equation ($[\eta] = K M_v^a$). The *K* and a in the equation have been determined in the literature and were adjusted by the degree of deacetylation (DD) according to the following equation:

$K = (1.64 \times 10^{-30} \times (DD)^{14})$ and $a = 1.82 - (1.02 \times 10^{-2}) \times DD$ where DD is expressed as the percentage, suitable for the determination of chitosan with over 60% DD. (Zhang Y et al, Carbohydrate Polymers 2006, 65, 229). Substituting the values of [η], K and a in the Mark-Houwink equation, ~$5.3x10^5$ Daltons is the viscosity average molecular weight of chitosan polymer.

Synthesis of FITC labeled chitosan (FITC-Chitosan) polymer

FITC is an amine reactive fluorescent dye. The isothocyanate group readily reacts with the primary amine groups of the chitosan polymer. The covalent attachment of the FITC to the chitosan polymer was carried out using a published protocol (Huang M et al; Pharmaceutical Research, 21, 344, 2004) with minor modification. The FITC labeling involved the following steps. First, 0.25% chitosan polymer solution was prepared in 1% acetic acid solution. Second, 6 mL of the chitosan polymer solution was treated with excess amount of FITC (dissolved in 6 ml of anhydrous ethanol, purged with N_2 gas) where the primary amine to FITC ratio was about 1:1.5. Under magnetic stirring condition, the reaction was allowed to continue for about a couple of hours in dark. Third, about 10 ml of 0.1 M NaOH was added to the reaction mixture to precipitate the FITC labeled chitosan polymer. Fourth, the precipitated FITC-chitosan polymer was centrifuged and washed repeatedly with a mixture of ethanol/water (70:30) till the washings were free of FITC (checked by the fluorescence measurements). Finally, the FITC labeled chitosan polymer was dissolved in 1% acetic acid and dialyzed against deionized (DI) water for about 48 hours.

The FITC-chitosan polymer was freeze dried for determining the FITC labeling efficiency by measuring the absorbance intensity of FITC-chitosan solution against standard solutions of FITC using UV-Vis spectrometer. The labeling efficiency was found to be 3.1 w/w % of FITC to FITC-chitosan polymer.

Synthesis of FITC labeled chitosan nanoparticles (FCNP)

Synthesis of FITC labeled ultra-small chitosan nanoparticles was carried out using TritonX-100/cyclohexane/n-hexanol/water in water-in-oil (W/O) microemulsion system. The crosslinker used was 25% stoichiometric ratio of tartaric acid (Bodnar et al, Biomacromolecules, 6, 2521, 2004). The carboxyl group of the dicarboxylic acid was reacted to the amine groups of

the chitosan by water soluble carbodiimide chemistry at room temperature. In a typical procedure, we prepared two separate W/O microemulsions (ME I and ME II). ME I was formed by dropwise addition of Triton X-100 to a mixture of cyclohexane (11 ml), n-hexanol (4 mL), a mixture of FITC–chitosan (2 ml as dialysed) and unlabeled chitosan polymer (2 mL). Upon magnetic stirring for about an hour, yellow-colored stable completely transparent microemulsion was formed. The ME II consisted of the activated tartaric acid crosslinker. The activation of tartaric acid was done following traditional water-soluble carbodiimide coupling agent, EDC where tartaric acid, EDC and NHS were combined in a ratio of 1:5:2 and reacted for 15 minutes (Damink L et al, Biomaterials, 17, 765, 1996). The ME II was then added dropwise to ME I under magnetic stirring and the crosslinking reaction was continued for 24 hours at room temperature. The FITC labeled covalently crosslinked chitosan nanoparticles were then collected upon breaking the microemulsion system by adding ethanol followed by centrifugation. The yellow colored nanoparticles were washed repeatedly (6 times) with ethanol. We applied brief sonication and vortexing during particle washing. About 3 ml of DI water was added to the centrifuged nanoparticle. Nanoparticles remained completely dissolved in DI water. To further remove any trace amount of surfactants and other reagents, nanoparticles were dialyzed against DI water for 48 hours. The dialyzed nanoparticle solution was then filtered using a 0.25 μm syringe filter, wrapped with aluminum foil and stored under refrigeration. Nanoparticle solution was freeze-dried and the yield was calculated to be about 10.5 mg/mL. The freeze-dried sample is easily soluble in DI water.

The FCNP particle size distribution was determined by Dynamic light scattering (DLS) measurements. Figure S 1 shows the particle size distribution in DI water in the ranges of 38 nm and 197 nm (**Fig. S1**).

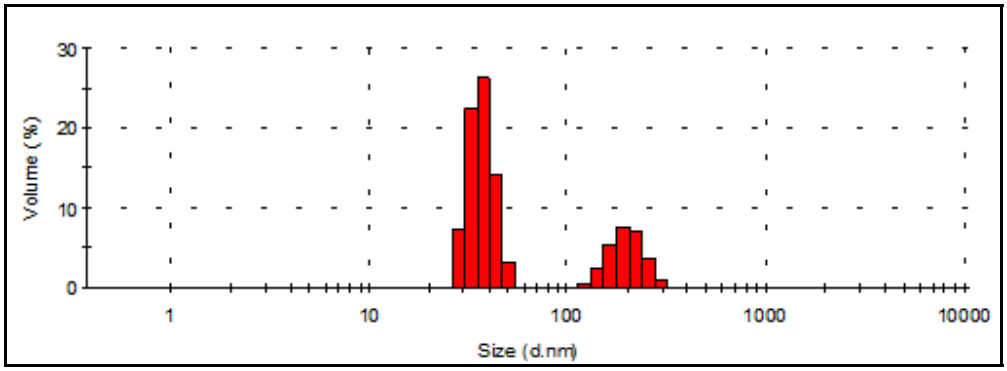

Fig. S1 : Particle size distribution of FCNP nanoparticles

The excitation and emission spectra of the FITC moiety in the FCNPs was determined by the spectrofluorometer. **Fig. S2** shows the fluorescence excitation (recorded at 519 nm emission) and emission spectra (recorded at 490 nm excitation) of the FCNPs recorded in DI water showed characteristic peaks of FITC.

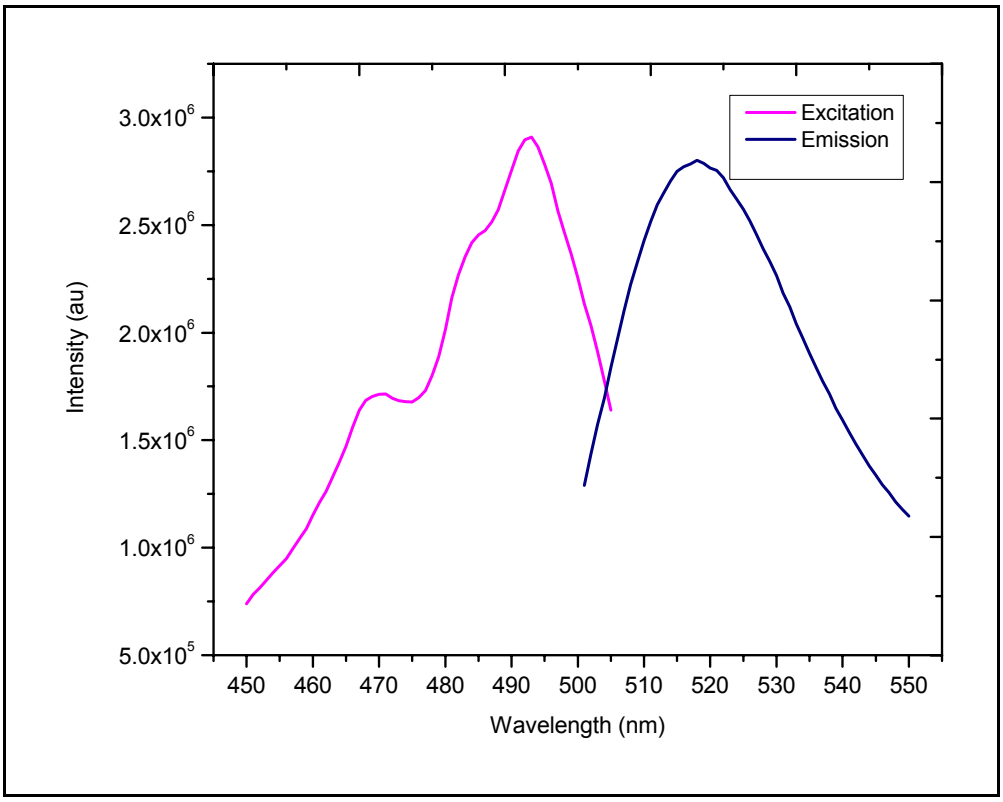

Fig. S2 : Excitation and Emission spectra of FITC moiety in the nanoparticles

Synthesis of aptamer-chitosan nanoparticles conjugate

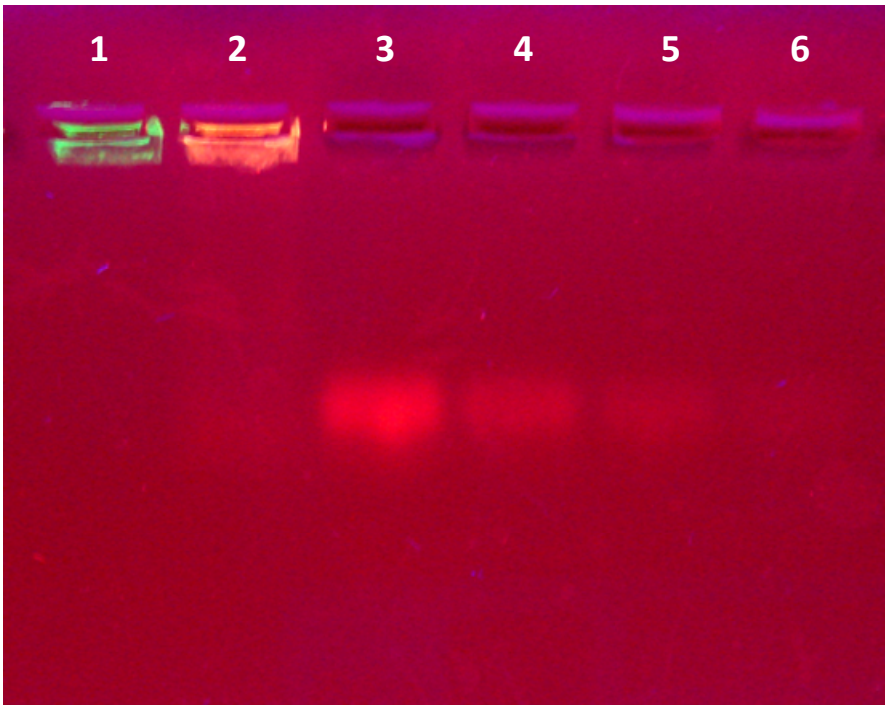

Fig.S3: Electrophoretic separation of sgc8c aptamer conjugated fluorescent chitosan nanoparticles (FCNPs) in 1% agarose gel for 25 min at 50 V in 0.5× TBE buffer (pH 9.0). The six lanes contain, from left to right, (1) FCNPs, 5.0 mg/mL; (2) sgc8c aptamer (5.0 μM) conjugated FCNPs, 5 mg/mL; (3) sgc8c aptamer, 5.0 μM; (4) sgc8c aptamer, 2.0 μM; (5) sgc8c aptamer, 1.0 μM; (6) sgc8c aptamer, 0.5 μM, respectively. The green color in lane 1 was derived from the fluorescent chitosan nanoparticles. Sgc8c aptamers have been stained by ethidium bromide with an orange color.

The aptamers selected were sgc8c, 5'-ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GA-3' and a library containing a randomized sequence of 41 nucleotides was used as a control. Both the aptamers were coupled with 5'-carboxyl modifier. The conjugation was carried out by adding 0.4 mg of EDC (~ 2 mM) and 1.1 mg of sulfo-NHS (~ 5 mM) to 100 μL of 5 μM carboxyl modified aptamer in 10 mM MES buffer (pH 6.5) and reacted for 30 minutes at room temperature. The excess reagents were separated from the activated aptamer by G25 Sephadex size-exclusion column equilibrated with 10 mM of phosphate buffer (pH 7.4). The concentration of activated aptamer was determined by UV-Vis spectrophotometer, followed by addition of activated aptamer to 0.1 mg/mL of FITC labeled chitosan nanoparticles at a final concentration of 0.05 mg/mL. The mixtures were then incubated

for 3 hours at room temperature. The gel electrophoresis (**Fig. S3**) result demonstrated that the majority (>90%) of aptamers preformed for conjugation had been covalently linked to chitosan nanoparticles. Therefore, no further purification was required.

Flow Cytometric Analysis

To demonstrate the targeting capabilities of aptamer-conjugated chitosan nanoparticles towards specific cells, fluorescence measurements were made using a FACScan cytometer. Approximately 1 $\times 10^6$ of each cell type was mixed with 100 μL of the nanoparitcles and incubated on ice for 20 min. After incubation, the cells were washed twice by centrifugation with buffer of 0.5 mL and resuspended in 0.2-mL volume of buffer. The fluorescence was determined by counting 10,000 events. The unselected ssDNA library conjugated with chiotosan nanoparticles was used as a negative control.

Cell Imaging

For confocal imaging, the treatment steps for cell incubation were the same as described in *Flow Cytometric Analysis.* Ten microliters of cell suspension bound with aptamer-conjugated chitosan nanoparticles were dropped on a thin glass slide placed above a 60× objective on the confocal microscope and then covered with a coverslip.