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Citation: Kim, A Ra, James J. Moon, Darrell J. Irvine, Sunghwan Jung, and Soong Ho Um. "DNA nanogel encapsulated by a lipid vesicle." In 10th IEEE International Conference on Nanotechnology, 454-457. Institute of Electrical and Electronics Engineers, 2010. © Copyright 2010 IEEE

As Published: http://dx.doi.org/10.1109/NANO.2010.5697779

Publisher: Institute of Electrical and Electronics Engineers (IEEE)

Persistent URL: http://hdl.handle.net/1721.1/79682

Version: Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

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Massachusetts Institute of Technology

Proceedings of 10th IEEE International Conference
on Nanotechnology Joint Symposium with Nano Korea 2010
August 17~20, KINTEX, Korea

DNA Nanogel Encapsulated by a Lipid Vesicle

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***Abstract-* DNA has been used as a new generic material due to its selectivity and intrinsic biocompatibility, to construct a variety of nano-architectures[1] such as cubic, tetrahedral, and even complicated origami. Recently, a 3-D hydrogel matrix of DNA has been manufactured at macroscopic scale[2] for biomedical applications as an extra-cellular matrix and a cell-free protein amplification platform[3]. To explore DNA hydrogel in a microscopic level, we report a novel method to produce DNA nanogel enclosed by a lipid vesicle. Nano-sized DNA hydrogels have been simply prepared by mixing precursors with DOPC lipid components under repeated sonications, following by nanometer filtering. After peeling off outer lipids using a lipid-chaotropic reagent (Triton X-100), we observe uniform nanogels entirely composed of DNA. With increasing the amount of DOPC lipids, the size of DNA nanogel has decreased. Our theoretical model based on equilibrium thermodynamics predicts such trend consistent with experiments. It indicates that DOPC lipid molecules energetically interfere with crosslink mechanisms among DNA units. DNA nanogel of well-controlled size may be incorporated with functional multi-modules and further applied to novel and advanced technological tools for in vivo diagnostics or therapeutics in preventive medicines.**

INTRODUCTION

Nucleic acid has been utilized as a structural material for nanostructures, due to its unique specificity and selectivity[1]. Since two decades, so many nucleic acids based nanomaterials have been produced. Representatively, Nadrian Seeman in NYU and his colleagues have manufactured novel DNA based nanoarchitectures[4] such as DNA tube[5], DNA cube[6], DNA crystal[7-8], DNA box[9], DNA origami[10] or so. They have furthermore exploited it for use of nanomechanical motor[11], a scaffold for regular patterning of gold nanoparticles[12] or protein molecules[13], DNA based nanocomputing device[14]. Recently, Dan Luo et al have demonstrated versatility of these novel DNA materials in advanced biotechnological applications such as a fluorescence-labeled DNA dendrimer and a DNA hydrogel. The DNA dendrimer can detect dangerous pathogen signals with higher sensitivity[15], and DNA hydrogel provides a protein amplification platform or a three dimensional extracellular matrix[2,3]. Generally, DNA gel has been formed by enzymatic crosslinking of DNA blocks. They are a complex of many oligonucleotides, in which each are complimentarily hybridized in order to construct three or four armed junctions as designed previously. To block the isotropic and bulky

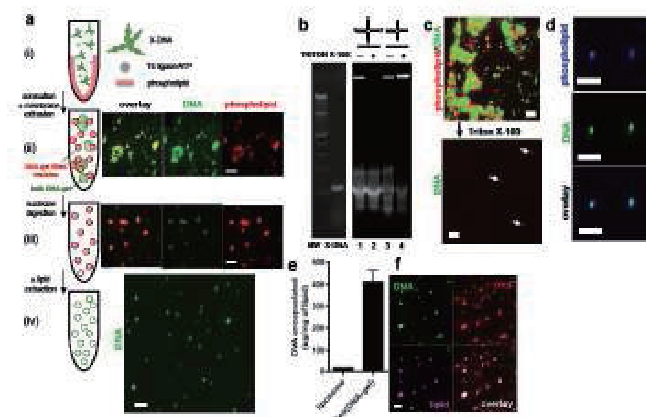


Fig 1. Synthesis and characterization of lipo(DNA-gel) particles. a, schematic view of synthesis and confocal images of reaction products at each stage (phospholipids traced by rhodamine-conjugated DOPC (red), DNA detected by SYBR I stain (green)). Scale bars 20 µm (ii); 2 µm (iii, iv). b, Gel electrophoresis analysis of DNA-gel particles formed with amine-capped or uncapped X-DNAs. Lane 1, lipo(DNA-gel) particles formed with amine-capped X-DNA; lane 2, amine-capped X-DNA particles following lipid extraction with triton X-100; lane 3, lipo(DNA-gel) particles formed with X-DNA; lane 4, crosslinked X-DNA particles following lipid extraction. c, DNA-gel products formed at step (ii) (top panel) and following step (iv) (lower panel) of the DNA gel particle synthesis performed using DOPG as the lipid template; white arrows point out rare particles collected following DOPC templating, scale bars 10 µm. d, Confocal images of maleimidefunctionalized lipo(DNA-gel) particles formed using membrane extrusion through 200 nm pore size membranes;blue DiD labeled lipid, green SYBR DNA stain; scale bars 5 µm. e, Comparison of ovalbumin protein encapsulation in ~250 nm diam. PEGylated DOPC:DOPG:MPB (4:1:5 mol%) liposomes vs. lipo(DNA-gel)s. f, Confocal microscopy image of ova-loaded DNA-gels showing colocalization of ova (red), DNA (green), and lipid (magenta); scale bar 5 µm.

gelation, DNA nanogel has been synthesized in the presence of templated lipid layers in nanometer thickness, which follow the successful achievement of Darrell J. Irvine group about the formation of PLGA based nanoparticulates with the help of lipid templates[16]. Although most current studies have shown interesting properties of DNA hydrogels, their general applications such as therapeutic reagents are still questionable due to the low versatility. Here, we report the development of DNA hydrogels in nanoscale and evaluate it with mathematical tool in order to confirm the formation of DNA hydrogel nanoparticulate. We also demonstrate their versatility by designing hydrogels with functional modules that allow controlled release of antigens for vaccine applications. It is highly expected that the DNA hydrogel nanoparticulates might be novel biomaterial resources for preventive[15] or therapeutic medicine.

978-1-4244-7032-7/10/$26.00 ©2010 IEEE

Materials and Methods

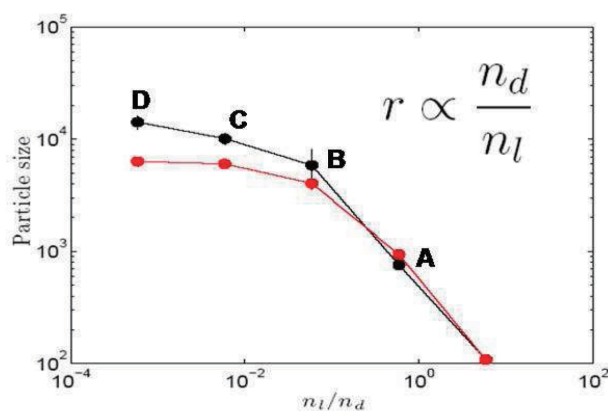
All chemical reagents and biological materials were commercially purchased from Sigma-Aldrich or Promega unless described. All oligonucleotides were commercially synthesized by Integrated DNA Technology. The sequence of four armed DNA junction called as X-DNA was designed and the X-DNAs were prepared and characterized by following the same procedures as described in our previous publications [2, 3]. Lipid sources were provided by Avanti Polar Lipid Co. X-DNA (1.67 mg) were mixed together with T4 DNA ligase solution containing 10 μL of T4 DNA ligase enzyme (Weiss unit: 3 unit per μL) and T4 DNA ligase buffer provided by Promega. They were added into dry lipid film containing a variety of lipid components in 1 mg. The lipid-DNA mixtures were strongly vortexed and put into a sonication probe. It was repeated 1 to 5 watts in power level under ice bucket and immediately extruded under nanometer sized membrane filter. After one-day incubation, it was first treated by an exonuclease and then centrifuged with 10% sucrose gradient in order to completely remove unreactants such as lipid or DNA debris. To remove lipid membranes covering the gel nanoparticles, it was further treated with either triton x-100 or phospholipase. Finally, DNA gel nanoparticles were collected using a high speed spin-down method. To determine loading and encapsulation efficiency, DNA nanogels with or without lipid coating were suspended into nuclease solution containing Exonuclease III (New England Biolab) and Buffer 1 at 37 °C for 1 1/2 hours. The combined solution was analyzed for quantification of fluorescence compared with standard curve of target drug in free solution. A standard curve correlating fluorescence and drug concentration was used to determine the amount of drug loaded into the DNA nanogel. Particle size and surface charge were determined by dynamic light scattering (DLS) using a ZetaPals (Brookhaven Instruments) ξ-potential and particle size analyzer. Particle formulations were analyzed in triplicate at a concentration of 10 μg/mL. The average values were from 5 runs of 30 sec each were used to determine both values. Particle morphology was also observed by laser scanning confocal microscope.

Results

Figure 1 showed the entire processing of DNA nanogel. Briefly, we mixed X-DNA and ligase precursors together with lipid components, and they were exposed to high-power mechanical forces in repeated cycles of 5 min. The resulting liposomal membrane entrapping X-DNA with ligase were extruded through membrane with nanosized pores and incubated over night, during which X-DNA blocks were ligated, leading to complete gelation.

A 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC) with neutral charge was used as a major lipid compoment. Using rhodamine-labeled lipid and DNA specific dye, SYBR I, we observed DNA nanogels with DOPC membrane, and subsequent triton treatment removed lipids, leaving nanosized-DNA nanogels intact(Fig. 1a).

To further confirm if complementary sequences of DNA blocks are essential for crosslinking inside lipid membranes, we tested crosslinking of DNA blocks designed with either non-complimentary or primary amine modified end groups. As expected, complementary DNA blocks admixed with ligase enzyme produced DNA nanogel which was too big to migrate onto agarose gel matrix(Fig. 1b). In contrast, no gelation occurred with non-complimentary or primary amine modified DNA block as they are unable to be recognized and ligated with other blocks(Fig. 1b). It was thus confirmed that complementary overhang and phosphate group on DNA block are necessary for crosslinking of DNA blocks. Next, size and shape of the final DNA nanogels were also evaluated with varying molar ratio of DNA block and DOPC lipid. With increasing lipid mass relative to that of DNA block, the size of DNA nanogel decreased rapidly(shown in the bottom of Fig. 2). Also, our theoretical curve on nanogel size is in a good agreement with experimental data shown in the top panel of Fig. 2. The theoretical modeling is as follow,



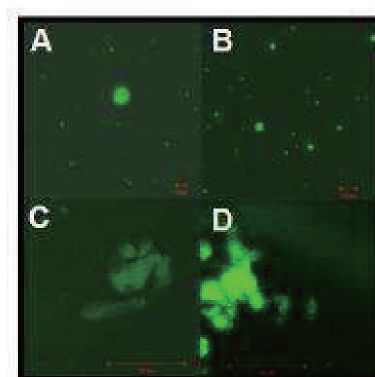


Fig. 2. Size evaluation (top) and confocal microscopic images (bottom) of DNA gel manufactured under varying DOPC lipid amounts from 0.001 to 10 mg. On the plot, nd and nl indicate concentrations of DNA and DOPC lipid, respectively. X-DNA is fixed at approximately 1.7 mg. With increasing DOPC lipid amount, the size of DNA product was getting smaller. The line in red color is the theoretical curve predicted from the relation between particle size and ratio of DNA and DOPC lipid.

Theoretical model

The formation of micron-sized vesicles is a complicated non-equilibrium process involving elastic deformation and chemical bondings of gel materials. Lipids cover the outer surface and DNA nanogels are filled inside. Not only energies associated with deformation of lipids but also chemical bonding of DNA gels should be considered. Following the previous work on the membrane dynamics, we further modified the model for our experiments.

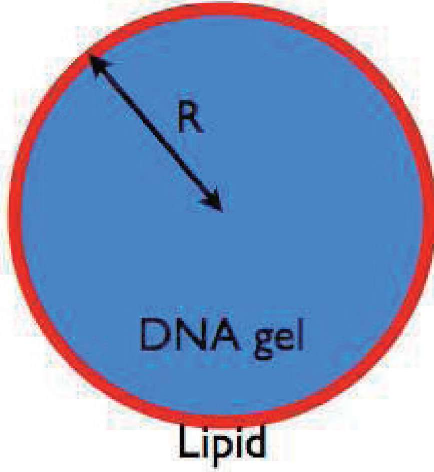


Fig. 3. Schematic picture of a nano-gel structure for mathematical model.

The total energy due to the area increase during the formation of vesicle mainly consists of elastic deformation and stretching. For the slow budding process, the dissipation energy is neglected because the energy dissipations in membrane and bulk fluid are inversely proportional to the time scale. Then, the total energy is composed of three parts: The bending and stretching energies.

$$E = n\frac{1}{2}\kappa S\left(\frac{2}{R}-\frac{2}{R_c}\right)^2 + n\sigma(S-4\pi R_c^2) + ne_{bond}\frac{4\pi}{3}R^3$$

where R is the radius of curvature, R_c is the intrinsic radius of curvature, and S is the surface area of a bud. When we assume a nano-gel is spherical, S becomes $4\pi R^2$. The last term is associated with a gelation where e_{bond} is the bonding energy per volume (N/m^2) and n is the number of nanogels at a given volume. There are several physical parameters to be determined in experiments [9]; the bending modulus κ is about 2×10^{-19} Nm and the stretching modulus σ about 3×10^{-7} N/m.

The total energy is a function of R and has a local minimum at a given R_c. This minimum value can be obtained by taking a derivative on the total energy with respect to R. The energy minimum is achieved when the bud radius becomes

$$R = \frac{-\left(\sigma + \frac{2\kappa}{R_c^2}\right) + \sqrt{\left(\sigma + \frac{2\kappa}{R_c^2}\right)^2 + \frac{4\kappa e_b}{R_c}}}{e_b}$$

When e_b is small,

$$R \approx \frac{R_c}{\left(R_c^2\sigma/(2\kappa)+1\right)}$$

The radius of bud will be maximum when $\frac{\partial R}{\partial R_c} = 0$, that is when $\bar{\sigma} R_c^2 = 1$. It concludes that R has a maximum of about 0.8 µm, which is close to our observation.

Number of lipid and DNA-gel molecules is given as

$$n_l = 2\frac{4\pi R^2}{a_l^2} \quad \text{and} \quad n_{DNA} = \frac{4\pi R^3/3}{a_{DNA}^3}$$

where a_l and a_{DNA} are the radii of single molecule of lipid and DNA, respectively. Mass of each material is proportional to the number of each molecule. Hence, we can simply get a relation

$$R \propto \frac{n_{DNA}}{n_l}$$

This relation is shown in the figure 2(a).

We next examined the flexibility of this synthesis in terms of lipid compositions compatible with particle formation and strategies to control particle size. First, we attempted to prepare lipid-capping DNA gel nanoparticles(=lipo(DNA-gel) using pure cationic lipids (1,2-Dioleoyl-3-trimethylammonium-propane, DOTAP), anionic lipids (1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol), DOPG), or PEGylated lipids (PEG-DOPE). Rehydration of DOTAP lipids with DNA-gel precursors led to instantaneous macroscopic aggregate formation and irreversible precipitation of the product, which could not be resuspended. Rehydration of DOPG with X-DNA precursor solution led to DNA-gel formation, but much of the DNA was segregated from the phospholipid (Fig. 1c), and low yields of particles were recovered following extraction of the lipid (data not shown). The mixtures of zwitterionic DOPC and anionic DOPG at a 4:1 molar ratio did permit DNA-gel particle formation. PEGylation of liposomes is commonly employed to increase their circulation time in blood and improve their transport through tissues[17,18]. We prepared lipo(DNA-gel)s using lipid mixtures containing 50 mole% of a maleimide-head group lipid (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide], MPB), and conjugated thiolterminated PEG to these DNA-gel particles post-synthesis. This approach successfully gave particles of similar size and yield as DOPC-only syntheses we thus tested whether DNA-gel particles could be templated by lipid mixtures containing 2KDa PEG-DOPE. Finally, we tested whether the size of the DNA-gel particles could be controlled in a manner similar to liposomes, by extruding the lipid/DNA-gel precursor complexes through filtration membranes immediately following sonication. Particle morphology and yield was similar to that observed with non-filtered samples, with lipids and DNA colocalized at the light microscope level (Fig. 1d).

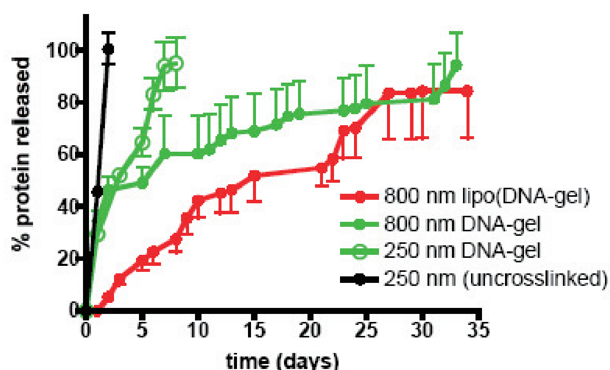


Fig. 4. Release kinetics of ova protein release from PEGylated DOPC:DOPG:MPB (4:1:5 mol%) lipo(DNA-gels) at 37 °C in serum-containing medium for particles with initial mean diameters of ~800 nm or ~250 nm.

A central application of interest for these hybrid phospholipid/DNA-gel materials is drug delivery of encapsulated therapeutic or imaging agents. To determine whether proteins could be loaded in lipo(DNA-gel)s without disrupting crosslinking/particle formation, we encapsulated a model protein antigen (ovalbumin, ova) in PEGylated lipo(DNA-gel) particles by including ova in the X-DNA precursor solution. Notably, the final quantity of protein encapsulated after particle synthesis and post-PEGylation was 25-fold greater than that entrapped within PEGylated liposomes of equivalent lipid composition, when compared on a per lipid mass basis (Fig. 1e). Using fluorescently-tagged protein, confocal microscopy confirmed the colocalization of ova with DNA and lipid in the particles (Fig. 1f).

We assessed the release of encapsulated proteins. Ovalbumin-loaded lipid-free DNA-gel particles of the size (~250 nm) released their protein cargo completely in ~1 week when incubated in serum-containing medium at 37°C (Fig. 4). However, larger ova-loaded DNA-gel particles initially 800 nm in diam. released protein over a much longer time-course, and 800 nm lipo(DNA-gel) particles continued releasing protein > 30 days (Fig. 4). Particles formed without ligase-mediated crosslinking by contrast released all of their cargo within ~2 days (Fig. 4), essentially matching the behavior of protein-loaded liposomes (not shown). We speculate that the vastly different protein release kinetics seen for 800 vs. 250 nm particles likely reflect the effect of particle size on both the kinetics of protein diffusion out of the multilamellar lipid/X-DNA network and nuclease diffusion into the network.

CONCLUSION

We demonstrated that only DNA based hydrogel nanoparticulate system is manufactured with the help of nanoscaled lipid capsule templates. We also developed a model to predict the size of nano-particle by considering associated bending, stretching, and bonding energies. This model shows a linear relation between the radius of nano-gel and a ratio of DNA and lipid concentrations, which is in a good agreement with experimental observations. X-DNA core enables modular insertion of functional or drug-binding components, proteins are encapsulated at very high levels per particle, and protein or small-molecule drug release from the particles can be sustained for up to 30 days. It is highly expected that DNA hydrogel nanoparticulate may be utilized for a variety of researches including nanobiotechnology, tissue engineering, bioelectronics, or so.

ACKNOWLEDGMENT

This work was supported in part by the NSF (MRSEC award DMR-02-13282), the Human Frontiers Science Program (award RGY0058/2006-C) and the Gates Foundation. Also, it was partly supported by NCRC program of the Korean Science and Engineering Foundation, which is being funded by the Korean government (Grant No. R15-2008-006-02002-0) and the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 20100007782; Mid-career Researcher Program).

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