Introduction to the revised application.

For this resubmission, we have extensively modified the previous grant application with omission of the original aim 1 and a greater focus on what originally was aim 2. Consequently, the entire research plan has been changed and new more focused aims, based on the original aim2, are formulated. One of the previous consultants, Dr. Shuster (biology, NMSU) has been recruited as a co-PI. He will bring his expertise in cell biology and in fluorescence imaging of cell related phenomena, as well as, the imaging equipment and cell culture facilities located in his laboratory. Because of very extensive changes, modifications are not identified in the text.

Two of the Reviewers pointed out that "the application contains several bright ideas" and that the goals "are enormous and worthwhile goals that could have many thousands of different potential applications." Nevertheless, all three Reviewers found the proposal "defocused" and impossible to finish. We agree with suggestion to split the proposal into two with greater focus, which should significantly increase the chance of getting maximum success. In this version, we focus on using hydrophobic nanoporous materials for cell transfection (new aim 1) and drug delivery (new aim 2). Regarding these parts in the original proposal, the Reviewers did not offer much of the constructive critique, probably because our preliminary data did not convince them and they were left to conclude that "the application of wettability switching mechanism in pores is not convincing". It appears from the comments that they did not appreciate the new proposed mechanism of **hydrophobicity switching** in intracellular transfection, in part because it suggests a new paradigm that the **transfection** can be achieved **without endocytosis**. To better illustrate how the mechanism works and confirm a high probability of success, we extended our preliminary data and provided detailed description of the proposed experiments and expected outcome.

To date, we made significant steps forward in confirming the universal nature of utilizing hydrophobic nanoporous membranes (HNPM) for cell transfection and came close to realization of a targeted drug delivery system based on multifunctional hydrophobic nanotubes (HNT).

1) We have previously described that HNPM membranes can be loaded by various cargo from small molecules (ionic and not), short nucleic acids and now demonstrate how plasmid DNA and proteins can be loaded as well

2) We have confirmed that HNPM indeed retain the cargo they are loaded with not only in a broad range of pH (from 2 to 14) but also in bovine serum (so far only at 10% BS), thus substantiating that 'the natural hydrophobic plug' is effective in various aqueous environment

3) We have previously demonstrated efficient transfection of HeLa cells from HNPM of the above mentioned cargo molecules: small ionic, DNA oligonucleotides… Now we have added BHK-21 and giant unilamellar vesicles (GUV). The latter result is a 'double-hit' illustration that endocytosis mechanism is not involved in transfection because not only the membrane cannot internalize but GUV does not have a cell machinery necessary for endocytosis.

4) Protocols for preparation of hydrophobic (inside) silica nanotubes (HNT) were optimized (with respect to various diameters and lengths and maximizing the amount of production) and various schemes of the outside surface of HNT modifications (with PEG and ligands) were developed. Protocols for HNT loading with different cargo were established

5) Additional means of HNT modification, including doping their walls with a dye and magnetic modification, were introduced.

These new (and old) preliminary data are outlined in section C.2. We hope these new results and a better planning of proposed study will increase enthusiasm about our proposal.

Specific comments to the Reviewers. Since the original aim 1 is omitted, no replies are given regarding that.

Reviewer #1.

This Reviewer apparently did not understand the proposed mechanism of drug delivery. His/her recollection presumes that "silica capsules (will be) filled with a hydrophobic drug.." is not correct. As we have demonstrated in the original preliminary data and now, various **charged** substrates are our primary transfection

molecules, including DNA and Rh. The sketchiness of our proposed experiments, to which the Reviewer also pointed out, has been hopefully rectified by more results provided and the research plan is now better focused. The interaction with consultants is outlined in section D.5.

Reviewer #2.

This Reviewer did not find our description of aims and the proposed experiments detailed enough. In particular he/she wanted the mechanism of drug delivery described along with the aims. He/she was concerned that the lack of concise description of the mechanism in the aims demonstrated a lack of clarity in my vision of the program. The reason for an admittedly very brief original formulation of the aims was in my concern over openness of that page to people beyond the review panel and I followed the instruction on preparing that page suggested to avoid proprietary information. Since that concern is now alleviated, a very detailed description of the mechanism of intracellular drug delivery is provided including a cartoon illustrating it. The Reviewer also wanted a comparison with other drug delivery systems and the cost feasibility of mass-production for such formulation. We have provided a comparison with other delivery methods and described one formulation realized in our laboratory in great details in section D.3.2.

Reviewer #3.

This Reviewer, besides agreeing with others on a too diverse set of aims, only commented that the original proposal provided “a list of possible uses of porous alumina” but “the alumina material itself has been studied extensively and is not a novel feature by itself”. We hope that focusing in the current proposal on cell transfection and drug delivery, as well as, a more detailed description of preliminary results, proposed experiments and anticipated outcome represents a convincing research proposal.

A. Specific Aims.

Increasing interest in applications of nanotechnology in biochemical sciences and in medicine is driven by unique properties of nanomaterials that can provide new opportunities for studying cell biology and in drug delivery. This project aims at investigating the possibilities of applying nanoporous materials for cell transfection and targeted intracellular drug delivery.

This **innovative** drug delivery system utilizes the effect of **hydrophobicity switching** illustrated in the cartoon of **Fig. 1**.

We hypothesize that alumina nanoporous membranes and silica nanotubes with multifunctional surface modification will be particularly effective in these roles through employing the hydrophobicity switching mechanism, in which their hydrophobic interior holds the loaded 'drug' but promptly releases it directly into the cytoplasm when in contact with the cell membrane. When phospholipids from the cell membrane decorate the hydrophobic walls, the drug is transfected directly into the cytoplasm without need for endocytosis. Such nanotubes equipped with specific cell recognition receptors should provide a universal drug delivery system with minimal side effects.

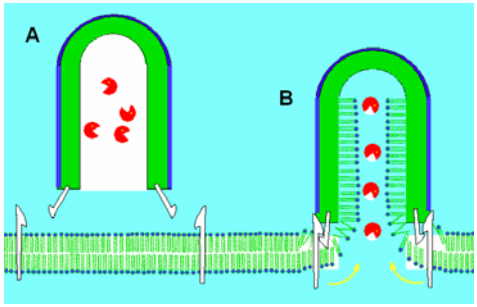

Fig. 1. Drug-loaded nanotube with hydrophobic interior (green) binds by ligands on its outside to the targeted cell surface and releases cargo after the cell's lipids decorate the pore walls upon binding.

To check this hypothesis, we will pursue the following specific aims:

<u>Specific Aim 1:</u> Optimize the cell transfection method based on hydrophobic nanoporous membranes (HNPM).

A very important tool in studying cell biology, such as interaction with new drugs, is the ability to transfect the cells with desired substrates: small molecules (*e.g.*, drugs), nucleic acids (*e.g.*, siRNA, antisense DNA or plasmid DNA), and proteins. Currently available non viral transfection reagents often are specialized for different substrates; but they allow translocation through cell membrane with limited efficiency and often pose undesired side effects by internalizing the drug carrier. The proposed new transfection mechanism is based on **hydrophobic nanoporous membranes** and requires **no endocytosis.** We plan to demonstrate its efficiency by pursuing the following goals:

1.1. Optimize the formulation for transfecting small drug molecules, oligonucleotides and polypeptides

1.2. Demonstrate the feasibility and optimize formulations for transfecting plasmid DNAs

1.3. Investigate the viability of transfecting large proteins

<u>Specific Aim 2:</u> Develop targeted drug delivery systems based on multifunctional hydrophobic nanotubes (HNT) and test their in vitro activity against a panel of cancer cells.

The concept of hydrophobicity switching will be applied to intracellular drug delivery. We propose to use silica nanotubes, which, in addition to their hydrophobic interior, will have functionalities on the outside for active cell-targeting in combination with PEG protection. The unique feature of this approach is again in **no need for endocytosis** as the mechanism for cell transfection. We intend to:

2.1. Investigate endocytosis of PEG-protected hydrophobic nanotubes: optimally shaped PEG-protected HNT should demonstrate minimal endocytosis and correspondingly low untargeted cytotoxicity.

2.2. Develop multifunctional modification of HNT, where outside surface has both PEG protection and ligands for targeted cell delivery.

2.3. Investigate the feasibility for targeted delivery of small molecules and oligonucleotides into cancerous cells in vitro

Our broad expertise in construction of such hybrid systems (see Preliminary Results) should allow for the successful development of these aims.

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B. Background and Significance

The method by which a drug is delivered can have a significant effect on its efficacy. Some drugs have an optimum concentration range within which maximum benefit is derived, and concentrations above or below this range can be toxic or produce no therapeutic benefit at all. Slow progress in the efficacy of the treatment of severe diseases suggests a growing need for a multidisciplinary approach in the delivery of therapeutics to target tissues, as well as, in alternative methods of treatment.

The objective of this proposal is to utilize hybrid nanomaterials in developing novel techniques for cell transfection and for targeted intracellular drug delivery. Both goals will utilize hybrid organic/inorganic nanoporous materials, where their surface is modified by a functional organic monolayer. Development of the physical aspects of drug delivery will be evaluated against various cancerous and normal cell lines. The findings will be also applied for assisting our collaborators in facilitating intracellular delivery of various substrates that they find difficult to perform in their research. The projects include delivery of anticancerous drugs and new estrogen receptor molecules, therapeutic nucleic acid delivery, and protein delivery. Unique properties of nanoporous materials offer new functionalities and approaches to common problems. Suitable utilization of these properties is in urgent need in biology and medicine.

Slow progress in the efficacy of severe diseases treatments, suggests an urgent need for a multidisciplinary approach to the delivery of therapeutics to targets in tissues. New strategies, often called drug delivery systems (DDS), address issues of controlling the pharmacokinetics, pharmaco-dynamics, non-specific toxicity, immunogenicity, and biorecognition using interdisciplinary methods that combine polymer science, pharmaceutics, bioconjugate chemistry, molecular biology, and physical chemistry.

Various DDS have been suggested to minimize drug degradation and loss, to prevent harmful side-effects, and to increase drug bioavailability and the fraction of the drug accumulated in the required zone. They include soluble polymers (Haag 2004; Rosler et al., 2001), microparticles made of insoluble or biodegradable natural and synthetic polymers (Agnihotri et al.; Soppimath et al., 2001; Vandermeulen et al., 2003), microcapsules, cells, cell ghosts, lipoproteins, liposomes (Muggia, 2001; Rivera et al., 2003), and micelles (Muller-Goymann, 2004; Torchilin, 2001). The carriers can be made slowly degradable, stimuli-reactive (e.g., pH- or temperature-sensitive), (Bae et al., 2003; Soppimath et al., 2005; Torchilin, 1993) and targeted (*e.g.*, by conjugating them with specific antibodies against certain characteristic components of the area of interest). Targeting is the ability to direct the drug-loaded system to the site of interest. Active targeting requires functionalization of the drug carrier's surface with ligands that are selectively recognized by receptors on the surface of the cells of interest. Since ligand–receptor interactions can be highly selective, this approach could allow more precise targeting of the site of interest. (Niculescu-Duvaz et al., 1997; Torchilin, 1985, 2004). Passive targeting can also be useful by employing the preferential accumulation of chemotherapeutic agents in solid tumors as a result of their enhanced vascular permeability compared with healthy tissue.

The mechanisms of release (active and passive) involve: (i) desorption of surface-bound /adsorbed drugs; (ii) diffusion through the carrier matrix; (iii) diffusion (in the case of nanocapsules) through the carrier wall; (iv) carrier matrix erosion; (v) a combined erosion /diffusion process. The mode of delivery can be the difference between a drug's success and failure, as the choice of drug is often influenced by the way the medicine is administered. Sustained (or continuous) release of a drug involves polymers that release the drug at a controlled rate through diffusion out of the polymer or by degradation of the polymer over time. Some believe that pulsatile release could be the preferred method of drug delivery, as it closely mimics the way the body naturally produces hormones such as insulin. This can be achieved by introducing functionality in DDS that responds to specific stimuli (e.g., exposure to light, changes in pH or temperature).

Delivering large molecules into the cytoplasm of animal cells without damaging the cells has been one of the strenuous challenges in drug delivery. Usually, the delivery mechanisms are designed to finely tune physico-chemical properties of the molecules to be delivered. There is no universal mechanism offered so far. For example, antisense oligos, DNA, Ribozymes, LNA, RNAi, and siRNA are polyanionic oligos are usually complexed with polycations, such as polyethyleneimine (Boussif et al., 1995), polylysine (Chan et al., 2000) or with cationic lipids (Thierry et al., 1993). Such complexes help protect these nuclease-sensitive oligos from

enzymatic degradation, and they are moderately effective for delivering the oligos into cells. However, such delivery reagents are generally rather toxic to cells and typically only work well in serum-free media. Advanced non-ionic antisense oligos, such as PNA (Simmons et al., 1997) and Morpholinos (Summerton, 1999), have been developed to overcome the problems of nuclease sensitivity and off-target effects. Because these oligos are non-ionic, they do not form complexes with the widely used poly-cations or cationic lipids; hence, they require other approaches, such as "protein transduction domain (PTD)" peptides, (Simmons et al., 1997; Moulton et al., 2003) including viral and excreted cellular proteins (Mann et al., 1991; Elliott et al. 1997) or synthetic analogs (Chassaing et al., 1997; Morcos 2001). In all cases, the delivery mechanism is usually associated with endocytosis, *i.e.,* when cells absorb material from the outside by engulfing it with their cell membrane. As a result, the current paradigm in DDS is that subsequent biodegradation is important for developing successful formulations, which is somewhat contradictory to the need for stability in the first place. Non biodegradable delivery systems did not receive as much attention because there was no mechanism of drug translocation included in such systems.

As we show in our preliminary results, there is an interesting, previously unrecognized mechanism for drug translocation through the cell membrane based on hydrophobically modified solid state nanoporous materials. Investigation of this novel approach is the topic of our suggested research.

C. Preliminary Studies

The proposed work relies on results from an ongoing project, which is in the last year of funding through a 3-year grant from the SCORE/NIH program. While studying application of nanoporous materials for biochemical sensing, we realized a potential for utilizing the hydrophobicity switching mechanism to designing a new universal cell transfection mechanism and intracellular drug delivery. It has become the main topic of this proposal.

C.1. Hybrid nanoporous materials.

Alumina nanoporous membranes provide a convenient template in nanotechnological applications. The membranes can be easily prepared by anodization of aluminum and can have diameters from a few nanometers to several hundreds of nanometers and controlled depths beyond 100 μm (Masuda et al., 1995, 1996, Jessensky et al. 1998, Li et al. 1998, 1999, Takmakov et al., 2006). Examples of homemade membranes prepared in 4% oxalic acid at 40 V and in sulfuric acid at 15 V are given in **Fig.2**. There is clear distinction in pore diameters: ~10 nm in A and ~ 60 nm in B. Commercial alumina membranes (of a lesser quality for pore diameter distribution) are also available from Whatman.

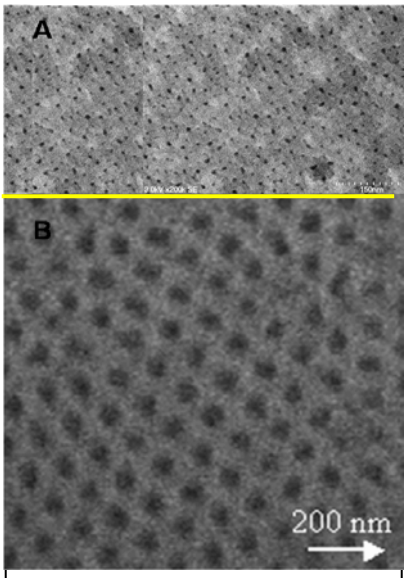

Fig.1 SEM images of homemade nanoporous membranes prepared by anodization of Al in (**A**) H_2SO_4 at 15Vand (**B**) in oxalic acid at 40V

Martin and co-workers have demonstrated how such membranes can be used as templates for synthesis of silica and polymeric nanotubes (Martin 1994). They also introduced the differential functionalization of silica nanotubes, where inner and outer surfaces can be modified by different organic monolayers (Mitchell et al., 2002). The method became attractive because inner voids can be used for capturing, concentrating, and releasing species ranging in size from large proteins to small molecules because tube dimensions can be easily controlled by the template synthesis (Mitchell et al., 2002; Kovtyukova et al., 2003; Okamoto et al., 2004; Son et al., 2005). The outer surfaces can be differentially functionalized with probe molecules to a specific target and/or environment.

Martin's group and others explored some possibilities of biomedical applications for such functionalized silica nanotubes (Gasparac et al., 2004; Hillebrenner et al. 2006; Son et al., 2007) but failed to identify the hydrophobicity switching mechanism as a means for cell transfection and drug delivery.

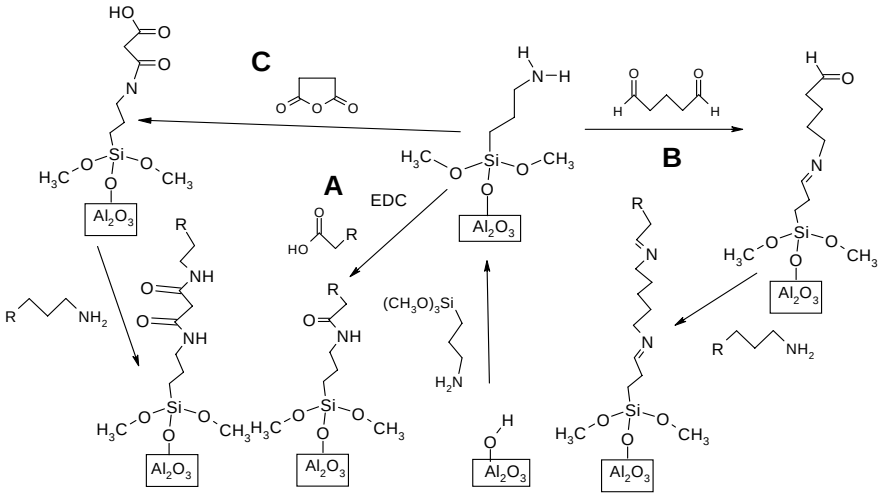

Fig.3. Typical schemes for immobilization of carboxyl (**A**) and amine (**B,C**) terminated molecules on the surface of oxides. Amination with aminoalkoxy-silane is followed by either (**A**) amide formation using EDC coupler or activation with gluteraldehyde (**B**) or maleic anhydride (**C**) and reaction with the desired amine.

All these applications require surface modification with desired molecules, including ligands and antibodies. Such surface modification can be achieved in a number of ways. The three most common ones used in our group (Smirnov et al., 2007a,2007b, Szczepanski et al., 2006; Vlassiouk et al., 2004,2005,2006,2008a, 2008b Takmakov et al., 2006a, 2006b, Rios et al., 2008). are shown in **Fig. 3**. Alkoxysilanes or chlorosilanes can be used for reacting with surface hydroxyls of either alumina or silica with similar efficiency. When aliphatic or fluorocarbon terminated silanes are used, it results in hydrophobic modification in

one step. More sophisticated modification can be achieved by using amine terminated silanes in the first step. Carboxylic terminated molecules can be bound to such aminated surface by EDC (N-(3-dimethyl-aminopropyl)-N`-ethylcarbodiimide) promoted coupling (A). If it is a fluorinated or aliphatic carboxyl, that also creates a hydrophobic surface. In order to bind amine terminated molecules (including peptides and amine modified DNA), the surface is activated by gluteraldehide (B) or maleic anhydride (C) to be able to react with the amino group of the desired molecule. The density of small molecules in a monolayer is very close to that of surface hydroxyls, ~ 4/nm^2. Larger molecules' densities are lower and for DNA appear to be limited by its gyration radius, *e.g.,* density of 0.05 nm^{-2} for ss-DNA 21mer can be reproducibly achieved.(Vlassiouk et al., 2004)

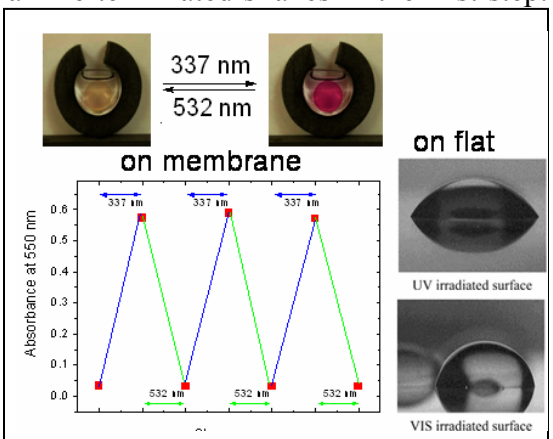

Fig. 4. UV irradiation turns spiropyran into the colored merocyanine form (top) rendering surface more hydrophilic (right). Vis irradiation recovers properties. Cycling can be repeated

C 2.1. Active hydrophobicity switching or smart surfaces

Here we present examples illustrating how complex such surface modifications can be constructed. Mixed monolayers that consist of both, hydrophobic molecules and the 'triggering' or receptor molecules that respond to either physical or chemical stimuli, allow construction of sensors. One of the mechanisms for sensing is based on active hydrophobicity switching. (Lee et al., 2001; Vlassiouk et al., 2008; Rios et al., 2008; Wanunu et al., 2007)

Light-sensitive surfaces and membranes. Optically active spiropyran molecule is less polar in its ground state spiro form. After excitation by UV light, it transforms into zwitterionic (polar) merocyanine form that renders the surface more hydrophilic (see **Fig. 4**). Irradiation by visible light switches spiropyran back to the spiro form. Mixed aliphatic/spiropyran modification of the membrane surface gradually transforms it from hydrophobic to somewhat hydrophilic upon increasing the portion of spiropyran, but when the latter is in the merocyanine (excited) form, this transition occurs at a lower concentration of the dye. In the range of intermediate concentrations, the membrane can be switched from its dry state, to a state where membrane is wetted by means of UV irradiation. The effect can be conveniently detected by ionic conductance though the membrane. (Vlassiouk et al., 2004) The membrane remains filled with water because the dry state is not achievable by spontaneous dewetting for pore diameters greater than 5 nm due to a high activation barrier but drying recovers their properties.

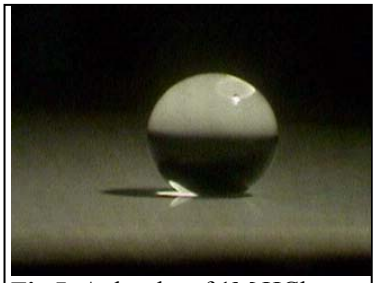

Fig.5. A droplet of 1M HCl on a top of fluorinated alumina membrane. The contact angle, θ>150^o, does no change with time.

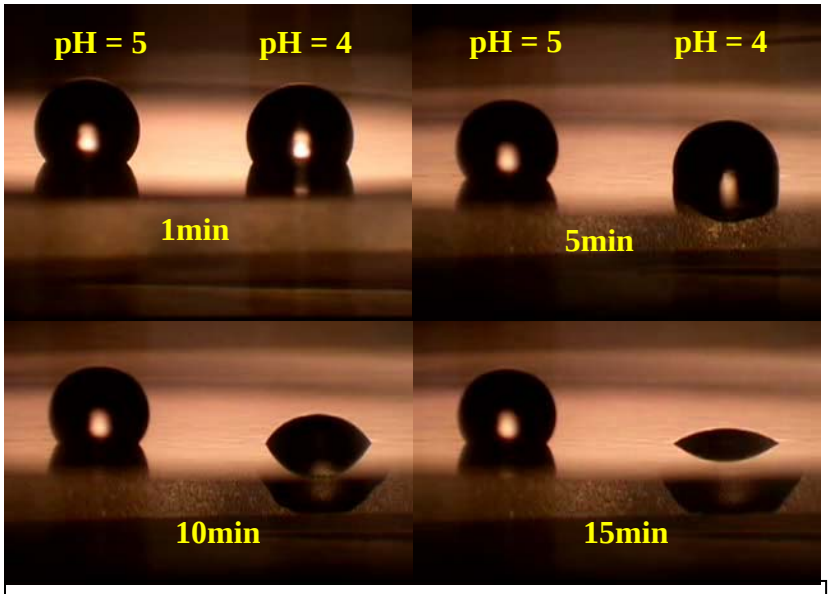

Fig. 6. Droplets of buffered solutions of different pH on a membrane with mixed amine/aliphatic modification. Droplets with pH>5 show very high contact angle indefinitely. Low pH droplets, as for the pH=4 shown, initially also have a high contact angle but the pores surface gradually wets by the buffer and the droplet passes through the membrane.

pH sensitive membranes. Different methods of hydrophobic surface modification produce varying quality hydrophobic surfaces but typically **a very broad range of pH stability, from 0 to 14**, can be achieved.(Smirnov et al., 2007a, 2007b) Hydrophobic membranes remain dry in this whole range (see **Fig. 5**) but they can be made responsive to a desired pH onset by using a mixed monolayer modification. (Smirnov et al., 2007a; Rios et al., 2008) **Figure 6** illustrates that mixed

amine/aliphatic modified membrane remains hydrophobic for high pH (pH ≥ 5). Droplets with a lower pH, despite initially showing a high contact angle, gradually wet the nanopores and pass through the membrane. It happens because the amines 'ionize' at low pH. Membranes modified with carboxyl-aliphatic mixed monolayers demonstrate an opposite behavior, they are hydrophobic at low pH but get wetted at high pH.

Bioanalyte-sensitive smart surfaces. Another example of active hydrophobicity switching employs biotin -

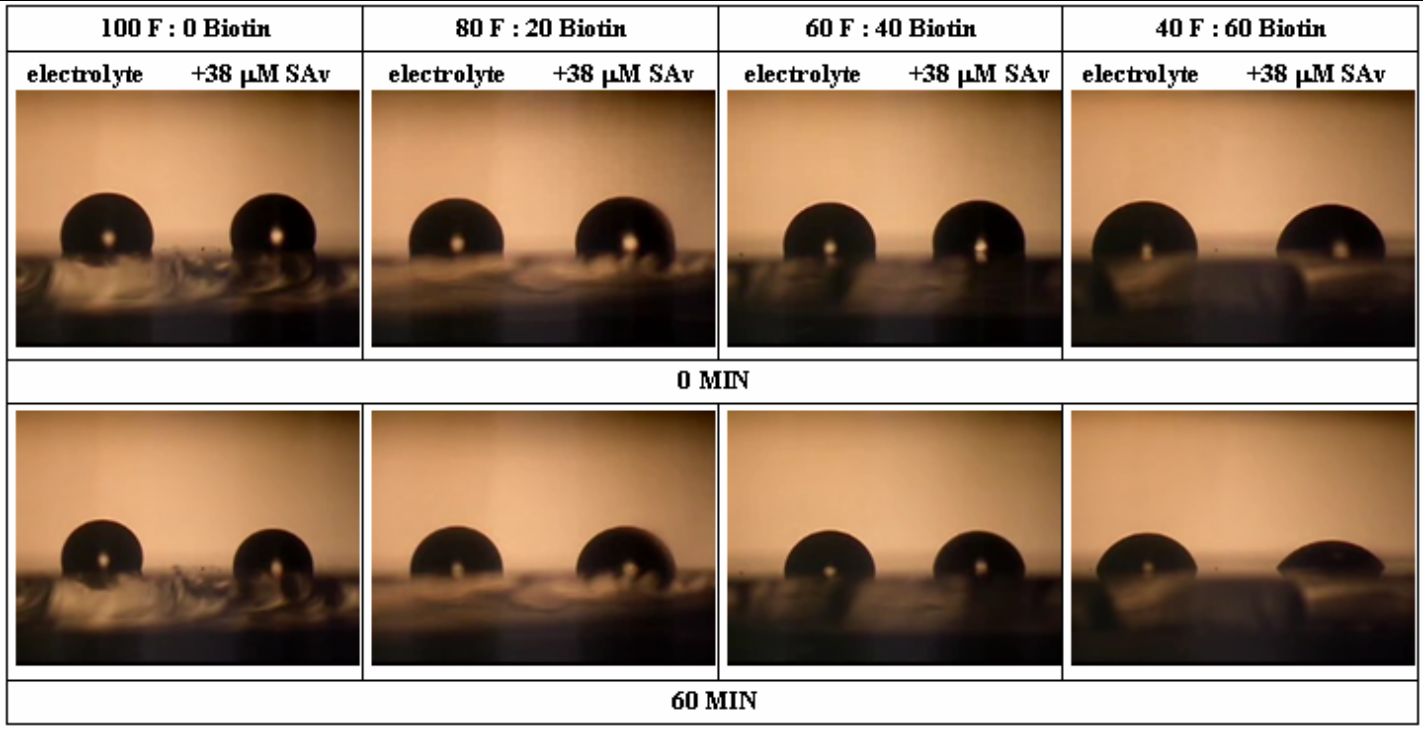

Fig.7. Droplets of a buffer and 38 μM SAV on surfaces modified with different proportions of fluorinated decane and biotin.

streptavidin (SAV) pair. (Rios et al., 2008) The biotin's binding site is buried quite deep into SAV. Thus, an effective coupling between them can be achieved only when the linker, by which biotin is attached to the surface, extends sufficiently above the neighboring hydrophobic adlayer. To ensure that and at the same time provide enough hydrophobicity to the surface, scheme A of **Fig. 3** was used in surface modification. The surface was first aminated by APTS and then linked to carboxyl terminated molecules using EDC coupling reagent. Mixtures of fluorinated decanoic acid and biotin shown on the left are used in different proportions. **Figure 7** demonstrates that, with increasing proportion of biotin, the contact angle with a buffer gradually declines. But, more notably, it is consistently lower if the buffer has streptavidivin in it. The difference is barely recognizable for the advancing contact angle but increases greatly for the receding one. The latter can be visualized by monitoring the droplets' shapes while they gradually evaporate. The contact angle initially equals that for advancing but decreases upon evaporation and eventually stabilizes at the value for the receding angle. Further evaporation no longer changes the droplets' shape and the contact angle.

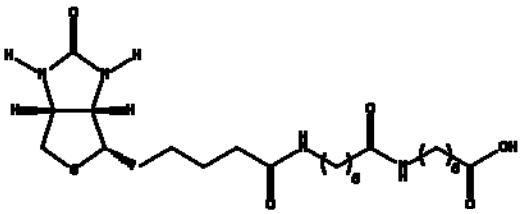

Table 1. Contact angles, θ, with and without SAV on flat surfaces modified with mixed fluorinated decane/biotin

Mixture for the monolayer formation	Contact angle w/o SAV, θ	θ with SAV
100F : 0Biotin	115 ± 3°	111 ± 3°
80F : 20Biotin	107 ± 3°	100 ± 3°
60F : 40Biotin	96 ± 3°	90 ± 3°
40F : 60Biotin	90 ± 3°	70 ± 3°
0F : 100Biotin	38 ± 3°	26 ± 3°

At the highest density of biotin, the contact angle change is visible even before evaporation. The

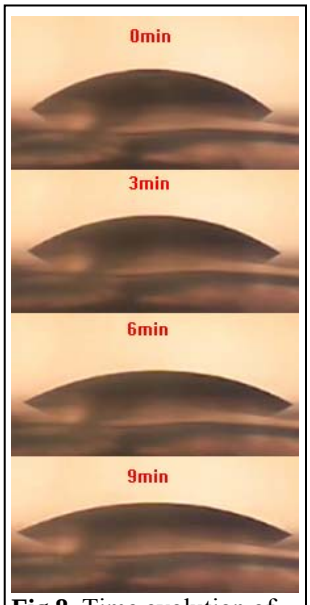

Fig.8. Time evolution of SAV containing droplet on biotynilated surface. Spreading not observed w/o SAV

droplet spreads, as shown in **Fig. 8**. The spreading is slower than that for pH sensitive surfaces because of a more hindered biotin/SAV interaction - the linker should be long enough not only to rich the binding site inside SAV but also to arch over the separation distance between neighboring binding sites. Even when the two requirements are fulfilled, as for the highest density of biotin with linker we use, binding itself requires time. With pH sensitive surfaces, high proton mobility produces an undetectable delay in establishing the equilibrium for expanding contact line on flat surfaces. But even there, buffer intrusion into the membrane pores extends over noticeable period of time.

C2.2. Hydrophobicity switching as a mechanism for cell transfection.

Uniformly modified hydrophobic nanoporous membranes (HNPM) and nanotubes with hydrophobic interior (HNT) stay dry in aqueous solutions but can be wetted by water either by applying a sufficient hydrostatic pressure or by decorating hydrophobic surface with amphiphiles such as detergents and phospholipids. This is the key idea in the proposed mechanism for cell transfection and drug delivery.

Effect of pressure. A hydrophobic channel/pore remains dry in aqueous solution if its diameter is small enough. It also means that whatever molecular cargo is inside of it, remains locked within due to this natural plug. The minimum external hydrostatic pressure at which water begins intruding into a hydrophobic nanopore depends on its diameter, D_{pore}, and the surface energy difference between water and the surface: (Smirnov et al., 2008b)

$$\Delta P = P_{ext} - P_{\text{int}} > \frac{4\Delta\gamma}{D_{pore}}.$$

The latter can be related to the surface energy at the water/vapor, γ, and the wall/liquid, γ_{wl}, interfaces via the Young equation:

$$\Delta\gamma = \gamma - \gamma_{wl} = \gamma\cos\theta,$$

where θ is the contact angle between water and the surface at the contact line. The pore diameter has to be less than 1 μm in order to sustain the atmospheric pressure. **Fig. 9** demonstrates how this reveals in the pressure dependence of ionic conductance through HNPM. When water and electrolyte intrude into membrane pores the ionic conductance increases. Smaller diameter pores require higher pressure. The method can be used for loading HNPM with desired molecular cargo directly from aqueous solutions but it is easier to achieve by using organic solvents with a lower surface tension.

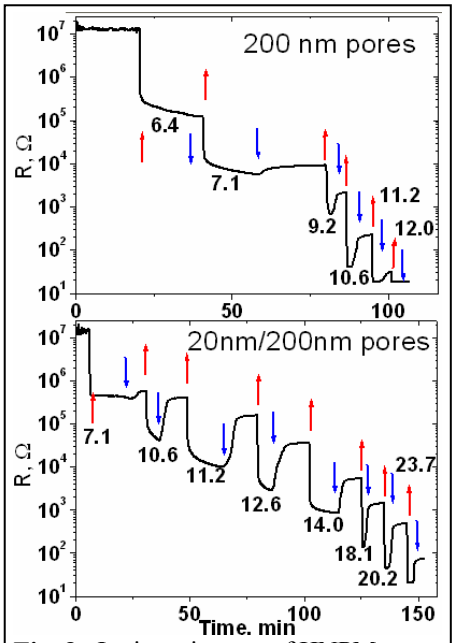

Fig. 9. Ionic resistance of HNPM vs. applied pressure in bars: arrows indicate on and off time. The difference is in both the intrusion pressure and the resistance recovery after releasing pressure.

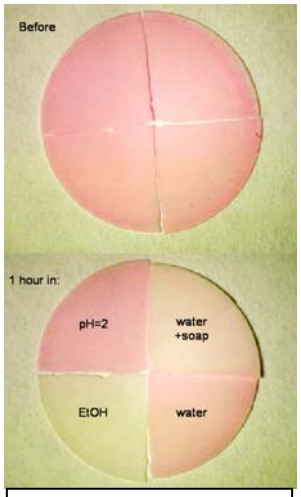

Fig. 10. Four pieces of HNPM loaded with RhB before and after 1 h in water, 0.5% SDS, EtOH, and HCl at pH=2

Effect of solvents. Hydrophobic nanopores and HNPMs provide a natural plug for water intrusion but are wettable by solvents with low surface tension. They can also yield to water intrusion by a detergent (Lahann et al., 2003; Steinle et al., 2002). The effect is illustrated in **Fig. 10**: HNPM was loaded with an ionic dye (RhB) using an ethanol solution. While pores remain dry, the loaded molecules do not leave the membrane. Water and acid of a gastric pH level can not intrude such HNPM either (**Fig. 10**). **The cargo inside hydrophobic pores can easily survive in the gastro intestinal tract and will be released only via additional stimuli that switch off hydrophobicity**. Dry membranes actually float in water or acid/base. Ethanol or an aqueous solution of a detergent have smaller contact angles on hydrophobic surfaces and can wash molecules away from the membrane easily. In ethanol, this happens instantly but in detergent, it takes over a couple of hours for the membrane and the detergent solution used in **Fig. 10**.

Effect of amphiphiles and cell membrane permeability. Steinle et al.(2002) also reported the effect of detergents but they found that detergents were wetting hydrophobic membranes at concentrations well below cmc (critical micelle concentration) - as low as 1 μM. Our results indicate that detergents effectively decorate

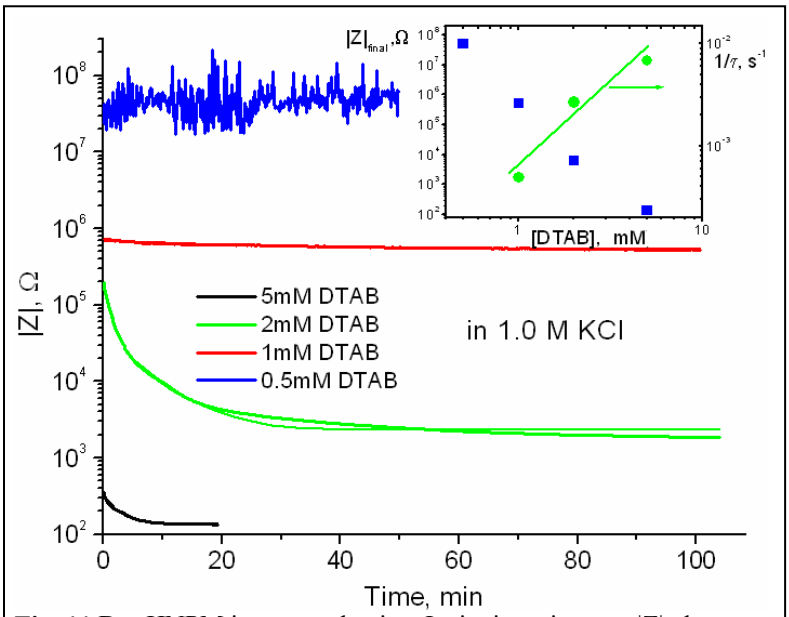

Fig. 11 Dry HNPM is nonconductive. Its ionic resistance, |Z|, drops from ~10^8Ω to below 200 Ω in a narrow range of amphiphile concentrations, [DTAB]. The electrolyte intrudes HNPM only at [DTAB] >> cmc~50μM. Both, |Z| and the relaxation time, τ, drop nonlinear with [DTAB] (inset).

hydrophobic pores only at concentrations well above cmc. **Figure 11** demonstrates it with dodecyltrimethylammonium bromide (DTAB) decorating hydrophobic walls of a membrane with 200 nm pores. The electrical resistance of the membrane remains unaffected by DTAB until 1 mM concentration, which is well above its cmc. The latter is less than 0.05 mM even at low ionic strengths and should be even less for 1 M KCl used here. The resistance drops to the value corresponding to that of totally 'open' pores only around 5 mM of DTAB – more than 100 times greater than its cmc. One can see that the ionic resistance at a particular concentration of DTAB gradually decays and the relaxation time, τ, shortens with increasing concentration of DTAB. The relaxation is not well described by a single rate (see, for example, 2 mM case and its exponential fit). Faster unresolved components cause a smaller initial value of the resistance after concentration of the detergent is increased. Moreover, the apparent relaxation rate, 1/τ, increases superlinearly with the concentration, indicating that the process is not diffusion controlled but likely involves concentration dependent association of the detergent on the walls. These results illustrate that a hydrophobic membrane or a single nanotube should keep its cargo dry unless the nanopore is placed in a zregion of a very high concentration of amphiphiles, such as, for example, lipids of the cell membrane. Our findings support this notion but contradict those of Steinle et al.(2002)

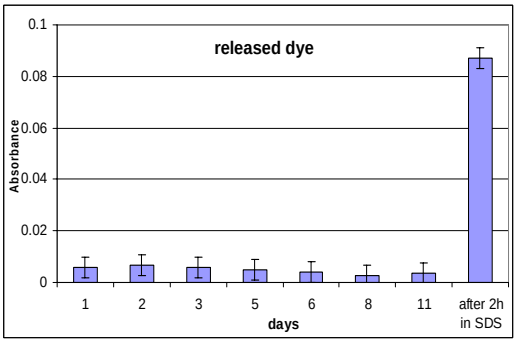

Fig. 12. The relative concentration of an ionic dye released from HNPM into 10% BS. Insignificant amounts were lost over 12 days. On the 12th day, the membrane was treated with 0.5% SDS to release all of the dye after 2 hours.

We hypothesize that this mechanism can be applied for **cell transfection and cellular drug delivery.** For a successful utilization, it is imperative that low concentrations of amphiphiles as free phospholipids, proteins, produce no effect on nanopore opening. Based on a crude estimate from **Fig. 11**, one may anticipate such a release time to exceed many hours at concentrations below cmc. To confirm it, we investigated a rate of dye release from HNPM in different environment. **Figure**

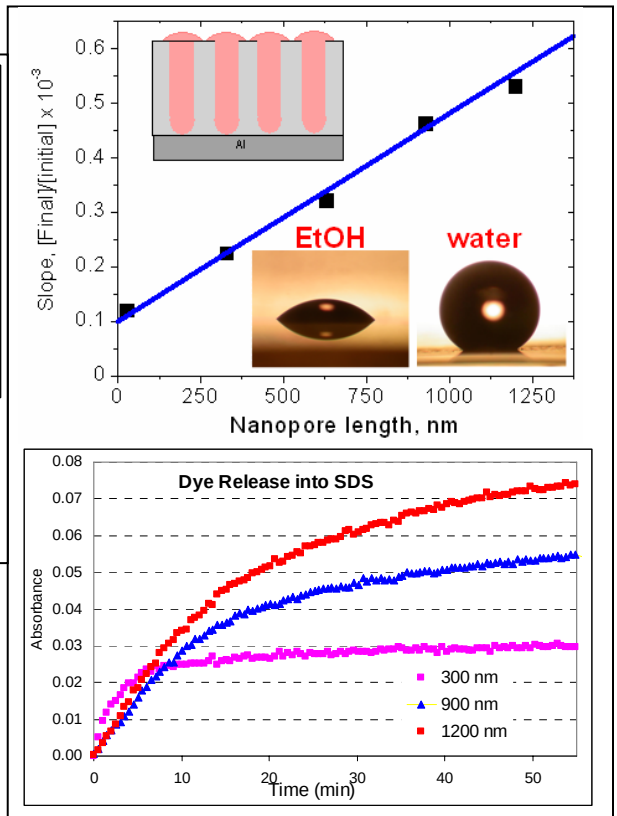

Fig. 13. The ratio of the dye concentration loaded and released from HNPM of different pore depths into 0.5% SDS. The slope of the linear fit is close to the anticipated. The nonzero intercept is due to extra dye sucked inside upon sample drying. The contact angle with EtOH on such HNPM, ~30°, (surface and pores wetted) is much smaller than that with water, ~140° (pores are dry). Kinetics below illustrate the slowing rates of dye release with pore lengths.

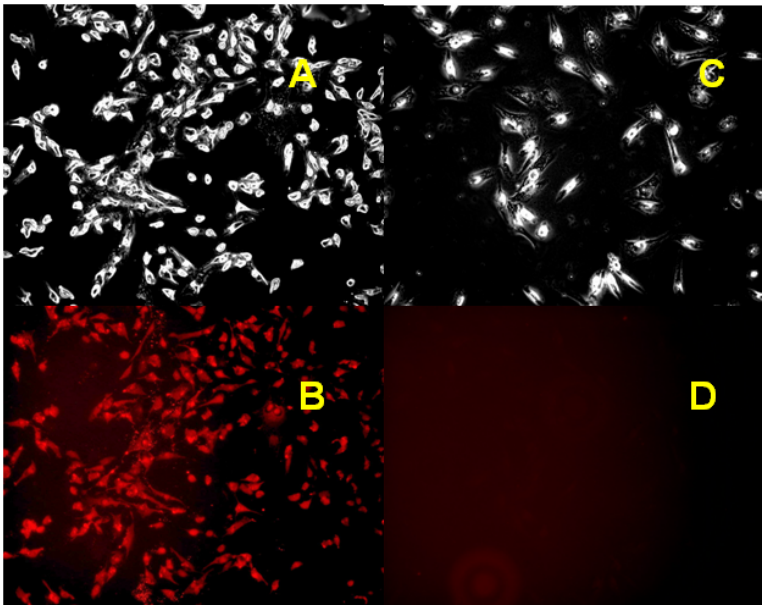

Fig.14. Phase contrast (A,C) and fluorescence (B,D) images of HeLa cells incubated in 10% bovine serum on the top of a hydrophobic (A,B) and untreated (C,D) membranes that were both loaded with RhB

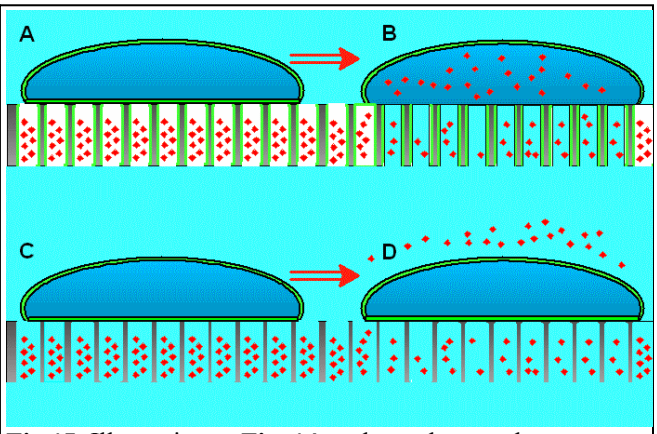

Fig.15. Illustration to **Fig. 14**: a charged cargo dye enters the cell only from a hydrophobically modified (green color) membrane. The hydrophobic pores of HNPM get wet only after their walls are decorated by phospholipids from the cell membrane allowing internalization of the cargo into the cytoplasm (A-B). The same cargo does not penetrate the cell membrane on its own or from unmodified pores (C-D)

12 illustrates that even in 10% bovine serum (BS) the dye remains inside the hydrophobic nanopores (60 nm in diameter) for weeks but is released within an hour (depending on the nanopores length) in 0.5% SDS. The amount of dye loaded into the nanopores relates nicely to the pores' volume and the concentration of the loading solution, as **Fig. 13** demonstrates. It confirms that all dye is released from the pores by a high concentration of the detergent. The release time depends on the pore depth and the detergent concentration. When the nanopores are not freed from the membrane, a thin layer of that solution remains on the membrane even after scraping off the excess solution. That causes a small amount of extra dye to end up in the pore and is detected as a nonzero intercept in that graph.

Figure 14 demonstrates the results of cell transfection using a hydrophobically modified commercial membrane with 200 nm diameter pores that was loaded with RhB dye. This dye is charged and is not easily internalized by cells (see **Figs. 14C** and **14D**). When HeLa cells were incubated in contact with such a HNPM, nearly all internalized the dye (**Figs. 14A** and **B**). The cartoon in **Fig. 15** illustrates the proposed mechanism: cells that were in contact with HNPM loaded by the dye received a high concentration of dye in their cytoplasm, while the control cells placed at the unmodified membrane surrounded by the same dye in solution and inside unmodified pores, showed essentially zero fluorescence. **Figure 14** provides the results for 20 hours of incubation but even after one hour there was a significant amount of fluorescence observed for the first group of cells. Moreover, there was a recognizable

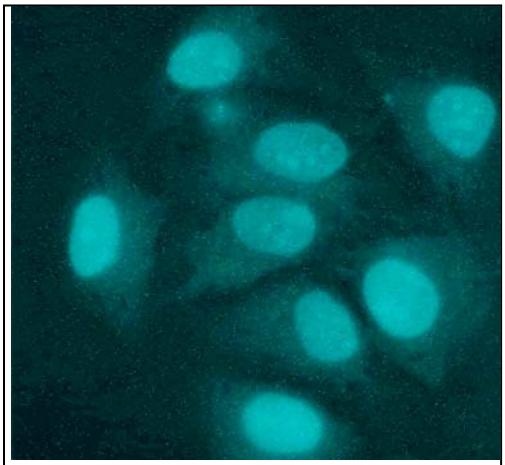

Fig. 16. Fluorescence images of HeLa cells incubated for 1 h in 10% bovine serum on the top of HNPM loaded with a 21mer DNA labeled by Cy5.

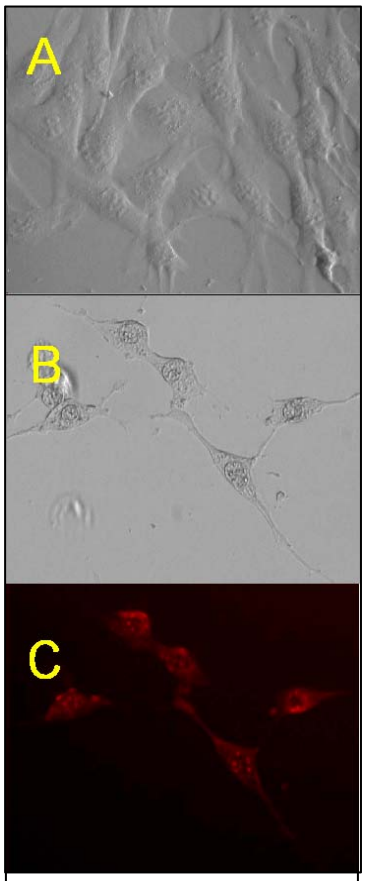

Fig. 17. Phase contrast (**A,B**) and fluorescence (**C**) images of BHK-21 cells incubated for 2 h on HNPM (**B,C**) with the same DNA oligo as in Fig.17. **A**) is the control .

stickiness of the hydrophobic membrane to the cells, which corroborates the interpretation that the lipid bilayer partially extends into the pores by decorating their walls, as illustrated by the cartoon in **Fig. 15**.

Similar results were obtained using fluorescently labeled DNA oligomers, as shown in **Figs. 16** and **17** for two very different cell lines: cervix cancer, HeLa, and hamster kidney, BHK-21. The loading of hydrophobic membranes with DNA has to be done differently because of its insolubility in organic solvents. We used the approach when the pores are first wetted with ethanol and then exchanged for a desired aqueous solution. The images clearly demonstrate that even after an hour the cells are transfected with DNA. One can recognize that in HeLa cells DNA is internalized in the nuclei (**Fig. 16**). The hamster kidney cells were not only transfected by the DNA but that oligo sequence was apparently toxic because the cells' morphology has altered significantly.

Delivering large molecules into the cytoplasm of animal cells without damaging the cells has been a challenge in cell biology. Even small charged molecules do not penetrate the membrane with ease. To the best of our knowledge, the **hydrophobicity switching mechanism** that we propose to employ for cell transfection, has not been discussed in the literature or explicitly realized before. It is possible that there have been some situations in which successful natural or artificial formulations for DDS have employed this mechanism without recognizing the contribution of the discussed phenomenon. The apparent ease with which it succeeds in our experiments leaves us wondering whether it is not employed in some natural mechanisms of cell invasion. Typically, various modes of endocytosis (when cells internalize the outside material by engulfing it with their membrane) have been considered as a rationale for designing new DDS formulations. The resolution of fluorescence microscopy is not sufficient for recognizing the more 'gentle' cell membrane puncture events like the one we propose. Since we can unambiguously eliminate the involvement of endocytosis in our experiments with hydrophobic membranes, this technique offers new research opportunities for studying cell biology. Some of these opportunities might include cell metabolism, cell division/fusion, ion channels, and others. **Universal mechanism** (various substrates can be transfected using the same HNPM) **of controlled delivery** (in both, the amount and the time) and **low cytotoxicity** (the transfection 'reagent' never eneters the cells) can be very useful traits in such studies.

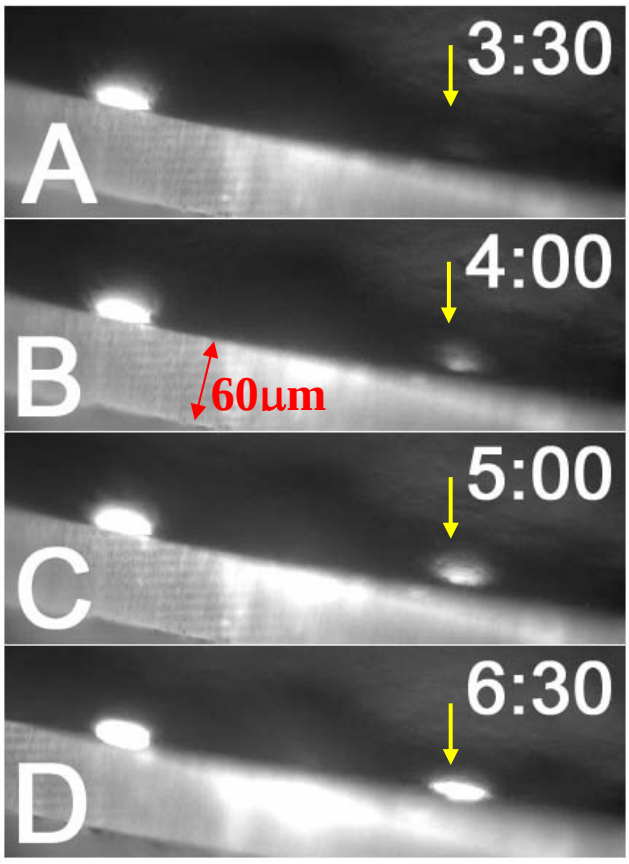

Fig.18. Time lapses of fluorescence images at the edge of a dye loaded HNPM with GUV diffusing towards its surface to attach and internalize the dye (see arrows).

C.2.3. Delivery into giant unilamellar vesicles.

Giant unilammellar vesicles (GUV) or giant liposomes represent a convenient model of cellular membranes that are 'free' of membrane proteins or/and intracellular machinery. Thus the endocysis mechanism for drug internalization cannot be utilized with GUV. The hydrophobicity switching mechanism, on the other hand, does not require endocytosis and should efficiently translocate any cargo through such a membrane. Our preliminary data illustrate that, indeed, GUV prepared by electroformation upon arriving at the surface of dye loaded HNPM, promptly attach to the nanopores and internalize the dye from the membrane. In **Fig. 18**, the membrane (60 µm thick) is oriented perpendicular to the image plane of the epifluorescence microscope. One GUV on the left is already bound and lit, while another lights up as soon as it touches the nanoporous surface. GUV were prepared using a liposome kit from Sigma (a mixture of L-α-Phosphatidyl-choline, stearylamine and cholesterol in 7:2:1 proportion). More detailed investigation is under way, where will vary the amount of cholesterol in the formulation.

C.2.4. Nanotubes for intracellular drug delivery.

We believe that these data represent the first unambiguous demonstration of a **new mechanism for cell transfection which does not require endocytosis** and thus should offer a **low intrinsic toxicity.** The mechanism should be **universal: independent of the substrate that needs to be internalized and insensitive to the environment.** One realization of this mechanism in drug delivery can utilize free floating nanotubes with hydrophobic interior (HNT). In particular, we intend to use silica nanotubes.

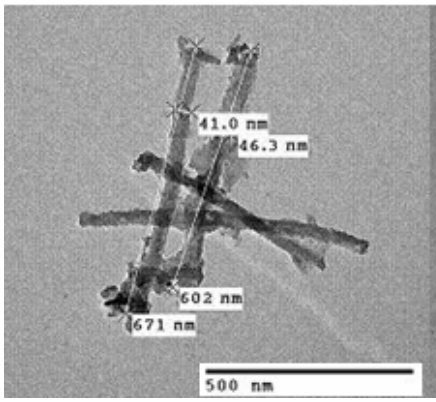

Fig.19 TEM of 50 nm silica nanotubes liberated from alumina template.

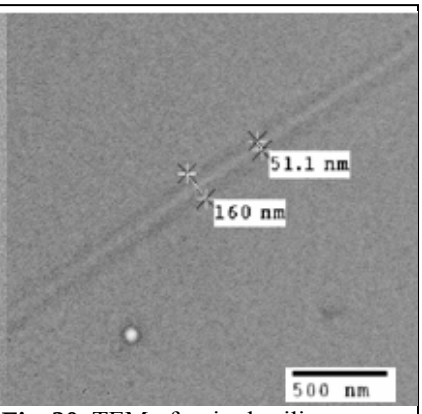

Fig. 20. TEM of a single silica nanotube prepared inside a commercial alumina membrane with 200nm pores

Silica nanotubes (NT) are easy to produce with desired dimensions using alumina templates. The surface modification of SNT is very similar to what we developed for alumina. Thus SNT is a very convenient choice for investigating this phenomenon, fine tuning to optimize properties and apply it for drug delivery. Martin's group was probably the first to demonstrate the method for differential modification of silica nanotubes. (Mitchell et al., 2002). It has been adopted by them and others for various applications including extraction and drug delivery. Various mechanisms of holding the load were suggested, including corking nanotubes (Chen eta al., 2005; Hillebrenner et al. 2006, Son et al., 2007) with nanoparticles and electrostatic interaction but no one has recognized the power of hydrophobicity switching mechanism yet.

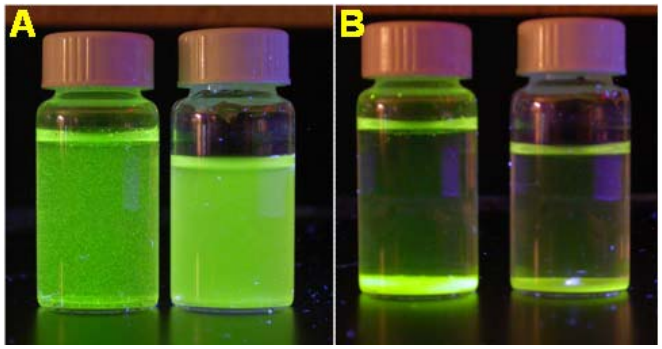

Fig. 21. A: HNT tubes (200 nm × 60μm on the left and 50 nm × 400 nm on the right) suspended in water. They were loaded with a dye before HNT liberation from the template by dissolution. **B:** The same HNT after precipitation.

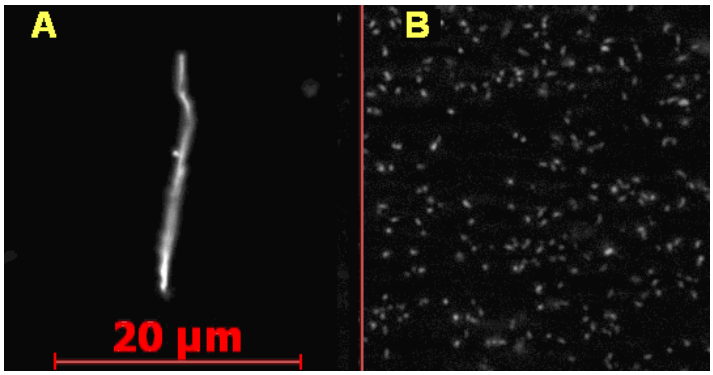

Fig. 22. Microscopic fluorescence image of the same HNT loaded with the dye, as in **Fig. 21**. **A**: a single ∅200 nm × 30μm tube, **B**: a collection of ∅50 nm × 400 nm tubes seen as dots.

Our silica nanotubes (NT), examples of which are shown in **Figs. 19-23**, are prepared by sol-gel the method inside of anodized alumina template. After hydrophobic modification of their interior, hydrophobic nanotubes (HNT) are freed by dissolving the alumina template in a strong acid or in a strong base. The outer surface can be modified by a different organic monolayer with desired properties. The inner diameter of the template can be varied up to 300 nm and defines the outer diameter (OD) of the nanotube. The inner diameter (ID) of NT can also be varied by the concentration of sol-gel and the number of times it is applied. The typical thickness of silica is on the order of 10 nm or more. The length of NT is defined by the depth of anodization in alumina template; it is

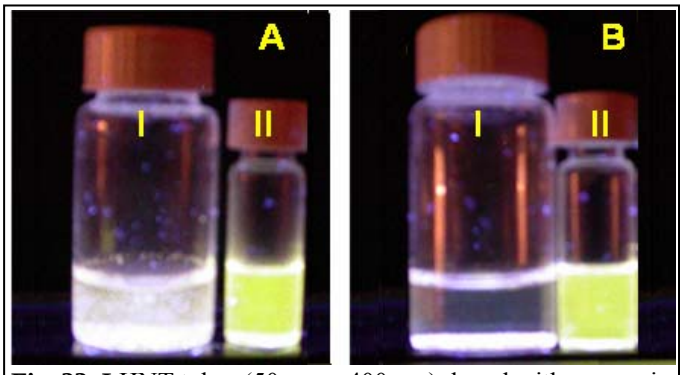

Fig. 23. **I**:HNT tubes (50 nm × 400 nm) doped with coumarin (blue luminescence), PEG modified outside and loaded with Rh6G dye. **II**: the same tubes exposed to 0.5% SDS, show Rh fluorescence. **A** –suspension, **B** – after precipitation.

about 650 nm in NT of **Fig. 19** and about 30 μm long in **Fig. 20**. The tubular structure is clearly visible in both cases.

The high quality of hydrophobic surface modification is demonstrated by the fact that HNT in **Fig. 21** were loaded with a dye before being freed from the template by dissolution. The dye remains inside the tubes during and after the template dissolution in phosphoric acid, confirming our previous assessment of a very efficient 'natural' plug from hydrophobic modification, without any need for artificial corking of such tubes for drug preservation and delivery. **Figure 22** shows fluorescence image of two 60 μm long dye-loaded HNT in water. Actually, freed HNT can be loaded with a dye with much higher concentration due the suction effect of **Fig. 13**. This high dye concentration causes it to form agglomerates with very low fluorescence yield. But when released into water by detergent or ethanol, the dye recovers its fluorescence, as shown in **Fig. 22**. Other important features of HNT in **Fig. 23** are that they are polyethylene glycol (PEG) modified on the outside and that silica walls of HNT are doped by a different dye, coumarin, which fluoresces in the blue and makes them distinct from Rh. The suspension shows blue fluorescence of coumarin but, after addition of SDS, green fluorescence of Rh becomes visible. Fluorescence of Rh6G released by the detergent, remains visible in the solution after precipitation of HNT (**Fig. 23B**).

Because of their unique organization, dimensions and surface modification, such nanotubes can be finely tuned and adjusted to a desired application. Other functionalities that we have also demonstrated are: a) mixed organic surface modification on the outside of HNT, including PEG and its combination with ligands such as biotin, folate and monoclonal antiobodies and b) magnetic modification inside of silica HNT.

D. Research Design and Methods

D.1. Rationale for the whole project and anticipated results.

The proposed experimental plan is organized into two specific aims; one aimed at applications in biology, another – in biology and medicine . In both goals we plan to utilize the unique properties of hybrid nanoporous materials: with hydrophobic interior that can hold a loaded cargo in aqueous environment of different constitution but release it promptly, when in the contact with the cell, across its membrane directly into the cytoplasm without need for endocytosis. The key idea is the previously unrecognized mechanism of delivery based on **spontaneous hydrophobicity switching** - when high concentration of phospholipids in the cell membrane decorates the hydrophobic interior of attached hydrophobic pores, rendering them hydrophilic, which guides the cargo directly into the cytoplasm. The goals are related via employing hybrid organic/inorganic nanoporous materials, where surface of nanoporous alumina or silica is modified by a functional organic monolayer.

D.2. Optimization of the cell transfection method based on hydrophobic nanoporous membranes.

D.2.1. Rationale and anticipated results.

A very important tool in studying cell biology, such as interaction with new drugs, is the ability to transfect the cells with desired substrates: small molecules (*e.g.*, drugs), nucleic acids (e.g., siRNA, antisense DNA or plasmid DNA), and proteins. Our proposed transfection mechanism is based on **hydrophobic nanoporous membranes (HNPM)** and requires **no endocytosis.** Thus far, we have demonstrated that cell transfection by small molecules and oligos can be successfully performed from HNPM. In order to develop the technique into a universal one, it has to be optimized for the maximum possible scenarios, including large plasmid DNA and proteins. Three generic conditions will be considered as individual goals and applied to substrates of interest to our collaborators. We plan to optimize the formulation for transfecting small drug molecules, oligonucleotides and polypeptides, demonstrate the feasibility and optimize formulations for transfecting plasmid DNAs and investigate the viability of transfecting large proteins.

D.2.2. Preparation of HNPM membranes for cell transfection.

The membranes for transfection of different substrates into the cells, such as small molecule drugs (doxorubicin, paclitaxel), nucleic acids (siRNA, antisense DNA, plasmid DNA) and peptides will be prepared as described above and illustrated in the sketch of **Fig. 245**. Polished 0.1-0.5 mm thick aluminum foil precut into desired shapes, after cleaning (first in acetone, then in 1M NaOH for 2 min) and electropolishing (in 3:1 of ethanol : perchloric acid at 10°C and 40 V), will be anodized at 4°C in 3% oxalic acid at 40 V. The solutions will be well stirred at all steps to ensure the uniformity of anodization. For a higher quality of membranes, the initially anodized layer (after 1 hr) will be dissolved in the stripping solution (1.8% w/v CrO_3, 6.0% v/v H_3PO_4 at 60°C for ~ 1hr) and anodized again to produce the final membrane will uniformly arranged even nanopores. The resulting nanopores diameter in the membrane, ~55 nm, can be increased to ~ 90 nm by treatment in 5% phosphoric acid for an hour at 30°C (Han et al., 2007). Alternatively, anodization can be performed in 5% phosphoric acid at 100V to get ~150 nm diameter pores. The length of the pores is defined by the duration of (final) anodization and will be varied within 300 – 3000 nm. The typical rate of the membrane growth is ~ 30nm/min for the above conditions in oxalic acid.

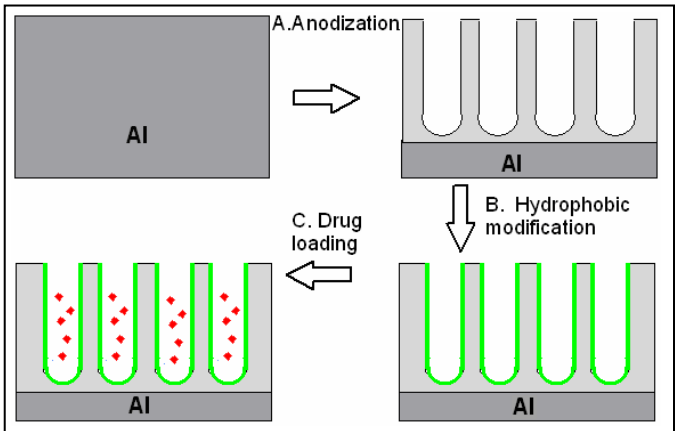

Fig. 24. Typical fabrication process of HNPM loaded with a drug. Anodization of aluminum produces an alumina membrane with the pore diameter and length defined by its voltage and duration, respectively (**A**). After hydrophobic surface modification (**B**), the membrane can be loaded with the desired substrate either from ethanol or by applying hydrostatic pressure to an aqueous solution (**C**).

Hydrophobic surface modification in step B of **Fig.24** can be performed using a number of ways identified in **Fig. 3**. As a default, we will use a one-step fluorination by 1H,1H,2H,2H-perfluorooctyltri-methoxysilane (Oakwood Products, Inc.) from ethanol solution (2% v/v silane + 2% v/v DI water in ethanol) for over 8 hr. The process is finished by washing in ethanol curing at 100°C for 3 hours. Such a modification renders the surface very hydrophobic and finishes the formation of HNPM. The quality of modification will be verified using the Sessile drop method of measuring water contact angle. For that purpose, purchasing of a DSA-20 EasyDrop Contact Angle Measuring Instrument is requested in the first year. The contact angle should be greater than 130°.

As **Fig.25** proposes, there are two parameters worthy of optimization: internal diameter, D_P, and the pores' length, H. When the membrane is in contact with the cell's lipid bilayer, because of many of such pores neighboring each other, the natural lateral mobility of lipids is hindered, leaving only those at the footprint of a pore mouth to decorate the walls of each pore. Presuming that the lipid monolayer density at the wall is the same as in the membrane, one arrives at a crude estimate for the height, $h \sim D_P/2$, of decorated (*i.e.*, 'wetted') depth of each pore. Preliminary experiments with 300 nm deep pore membranes suggest that transfection from them is just as effective as from 60 μm long pores. If the pore length is optimized so that $h \sim H$, the amount of load internalized by cell can be quantified with high precision and used with minimal waste. It is likely that the lipid density on the walls is noticeably less than that in the cell membrane and/or they can redistribute between the pores making h greater than $D_P/2$. Experimental optimization would be the straight forward approach to verify that. The pore diameter can be also varied, *e.g.* broadened, to

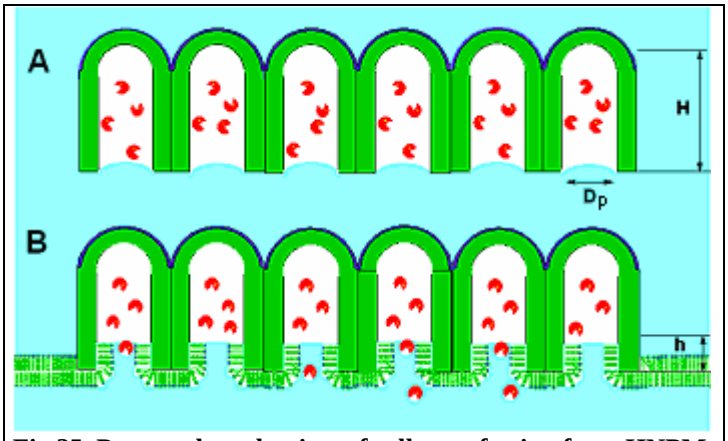

Fig.25. Proposed mechanism of cell transfection from HNPM: Hydrophobic modification (green) inside can be decorated by cell lipids only to a certain depth due to limited number of molecules at the tube's 'footprint'.

accommodate larger sized substrates for transfection.

D.2.3. Membrane loading for transfection of small molecules.

D.2.3.1. Loading from ethanol solution. Ethanol soluble substrates (*e.g.* doxorubicin) will be loaded into the nanopores from ethanol solution. Typically, a HNPM membrane will be immersed into 10 μM or higher concentration solution for 10 min and, after wiping off the excess, the membrane will dried at ca. 50°C for 15 min. Finally, the membrane will be washed in copious amount of DI and dried again. The resulting surface should recover its original hydrophobic appearance of the top coverage and retain the substrate inside.

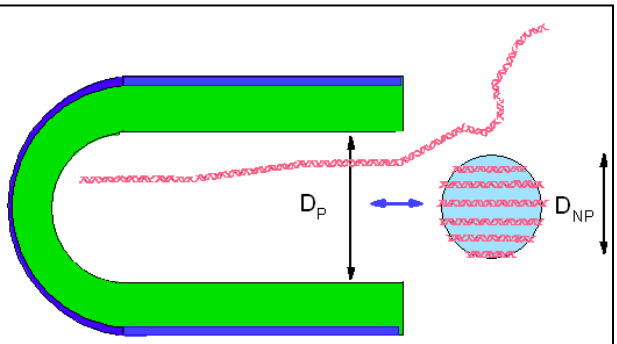

Fig.26. Illustration of nanoparticle (NP) bound plasmid DNA (red) and the nanopore entrance. Diameter of NP with plasmid, D_{NP}, has to be smaller than D_P. Unbound plasmid, shown for comparison, does not fit into the pore

D.2.3.2. Loading from aqueous solution. Substrates that are insoluble in organic solvents, such as short nucleic acids (antisense DNA oligos, siRNA), and peptides will be loaded from aqueous solutions. HNPM will be first wetted with ethanol and then immersed into DI water for 30 min to replace alcohol with water. Still wet with water membrane will be covered with the aqueous solution of substrate for 30 min. After wiping off the excess liquid, the membrane will be dried as described above at 40°C for 30 min, washed in copious amount of DI water and dried again. The resulting surface should recover its original hydrophobic appearance on the top and retain the substrate inside. Plasmid DNA, due to its large size, and some proteins cannot be loaded using this protocol, and require special approach.

D.2.4. Membrane loading by plasmids for transfection.

Large size of plasmids, 1-50 kb, prevents them from entering into the nanopore completely; at best, only partial entry can be achieved, as illustrated in **Fig. 26**. Indeed, the extended length of 6 kbp plasmid exceeds 2 μm. Cell transfection from HNPM in this case would not occur. Two protocols will be used and optimized for compacting the plasmids and loading them into the nanopores: using polyamines and positively charged solid nanoparticles. Since 'compacted' in such a way plasmid complexes also demonstrate increased internalization by cells, their transfection efficiency will be compared with and without HNPM.

D.2.4.1. Polyamines based plasmid packaging. Polyamines are known to induce shrinking of plasmid DNA into small nanoparticles (Fink et al., 2006). Polylysine, pLy, (110 kDa) forms collapsed complexes with 6 kb plasmid as beads of less then 20 nm diameter, when the ratio of lysine/base ~ 4 (Chan et al., 2000). This size is sufficient for the typical 50-200 nm diameter pores of our HNPM. Slightly less efficient contraction is achieved with spermidine, with which a similar plasmid is shrunk 'only' to 80-100 nm diameter beads. This diameter is nevertheless smaller than 200 nm of the available pores and thus will be investigated as well. DNA complexation with polylysine (~100 kDa or ~200kDa; Sigma) will be carried out by adding 2 μg of plasmid DNA to various amounts of pLy in a final volume of 100 μL HBS buffer (Hepes-buffered saline: 0.15 M NaCl, 20 mM Hepes, pH=7.4) to obtain a range of Ly/Nu ratios (3-5), vortexing for 10 s, and incubating for 30 min at room temperature before use. The effect of pLy counterions will be also performed since it was reported that trifluoroacetate and acetate ions have apparently a better effect on compacting the plasmid with ellipsoidal and rod-like shape, respectively (Fink et al., 2006). Complexation will be monitored by gel electrophoresis and TEM. Similar approach will be used with spermidine.

D.2.4.2. Nanoparticles based plasmid packaging. Large plasmids can also be wrapped around positively charged nanoparticles (NP), such as alumina or aminated silica NP (Hen et al., 2003). Appropriate NP diameter, D_{NP}, will be used for different sizes of plasmid. The surface charge on NP should exceed that of the plasmid not only to capture it in complete on the surface but to be in excess for preventing neutralized NPs from aggregation. At the same time, D_{NP} should be less than the pore diameter, $D_{NP} < D_{NP}$, for easy entrance into the

nanopores (see **Fig.26**). With a typical surface charge density, $N_C \sim 4e/nm^2$, and using the surface area of NP, the minimum NP diameter can be estimated:

$$\text{NP Charge} = N_C \pi D_{NP}^2 > \text{Plasmid Charge},$$

which for 6 kb plasmid is on the order of 35 nm. The plasmid (at concentration 1μg/1μL), such as a GFP encoding vector, will be incubated with 40 nm diameter alumina NP to the final ratio of plasmid/NP < 1 in sterile water for 30 min. Complexation will be monitored by gel electrophoresis and TEM. **Figure 27** illustrates a representative electrophoresis gel of titrating 6kb plasmid with 40 nm alumina NP, confirming the quality of binding.

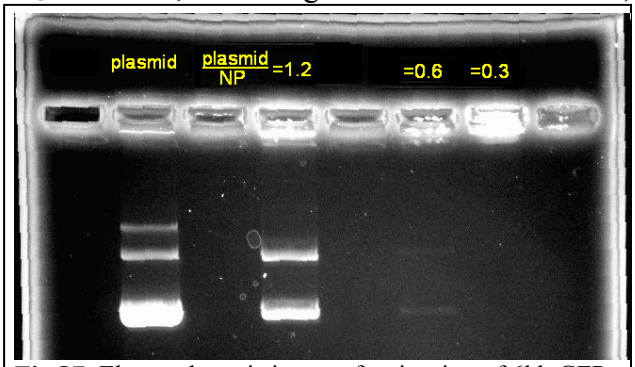

Fig.27. Electrophoresis images for titration of 6kb GFP plasmid binding to alumina NP of 40 nm diameter. The brightest spots correspond to the supercoiled form.

D.2.4.3. Loading of packaged plasmids. Packaged plasmid after mixing with PBS buffer to 5mL of a final concentration 0.2μg/μL, will be placed atop of a prewetted HNPM membrane for 2 hours prior to transfection experiments. After that, the supernatant will be collected, membrane briefly washed with PBS and dried. Hydrophobicity of the dry loaded membrane will be checked by the Sessile drop method. If the hydrophobicity is not fully recovered, additional washing in PBS (with drying) will be performed until its full recovery.

D.2.5. Preparation of partially hydrophobic nanoporous membranes (PHNPM) for cell transfection.

Transection of proteins into cells (protein delivery) is notoriously difficult but could drastically expand the techniques for manipulation in cell biology. It represents a powerful tool for experiments in live cells including studies of protein-protein interactions, protein interference with blocking antibodies, intracellular trafficking and protein or peptide biological functions. Many available reagents dedicated to the protein delivery allow crossing of the plasma membrane but major disadvantages for these reagents include a) weak release of the delivered protein into the cytoplasm, b) inhibition of the efficiency by serum, c) cytotoxicity of the reagents.

Delivery from HNPM can overcome these shortcomings but requires some tweaking. It concerns not the size of proteins (most proteins easily fit into the typical nanope size of 60 nm) but their sensitivity to the environment. Many proteins ceaze functioning after they were dry and thus keeping them in dry hydrophobic nanopores before delivery may become challenging for recovery of their native form afterwards. Moreover, proteins often stick to hydrophobic surfaces after a long exposure. (Krishnan et al. 2005) To overcome these shortcomings, we will use partially hydrophobic nanoporous membranes (PHNPM) as illustrated in **Fig.28**. Such membranes will be constructed by a short initial anodization and hydrophobic modification to form shallow (100-200 nm) HNPM, as described above in D.2.2. The second anodization (step **C**) extends the pore length, and allows different modification. This portion will be PEGylated using 2-[methoxy(polyethyleneoxy]trimethoxysilane (Gelest, Inc.) to render the surface hydrophilic but lipophobic at the same time. As **Fig. 29** illustrates, such membranes have a vapor plug outlined by the hydrophobic belt at the entrance, which retains the protein 'solution' in its native form inside the pore. Loading of such PHNPM membrane by proteins is almost identical to that described in

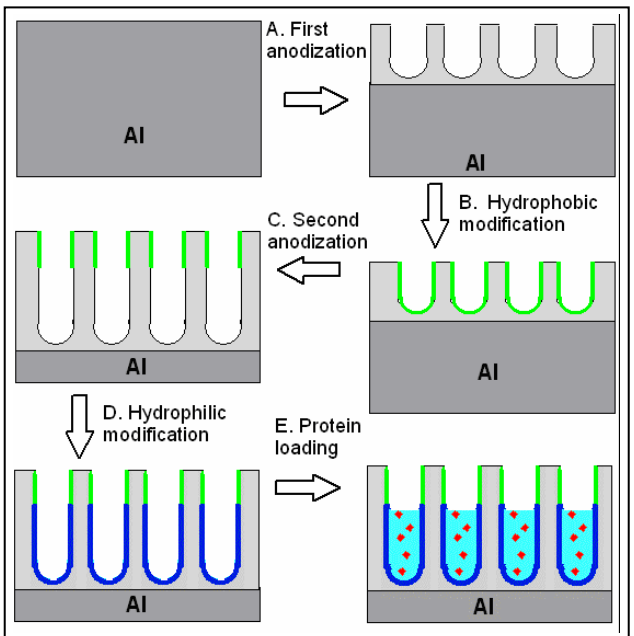

Fig. 28. Fabrication process of PHNPM for protein transfection. Anodized alumina membrane grown on aluminum to a short (~ 100 nm) length (**A**); is hydrophobically modified inside (green) (**B**). The second anodization extends the depth (**C**) that is PEG modified (**D**) suitable for protein loading from aqueous solution by applying high pressure (**E**).

D.2.3.1. for loading from aqueous solutions. The only difference is in the duration of drying, which will be shorter to preserve moisture in the hydrophilic part. The length of the hydrophobic belt, *h*, will be optimized to balance the ability to maintain the protein cargo and the easiness towards releasing it at the cell membrane. The value of *h* is anticipated to be on the order of the pore diameter, as discussed above. Optimization will be necessary also for the duration and temperature of drying.

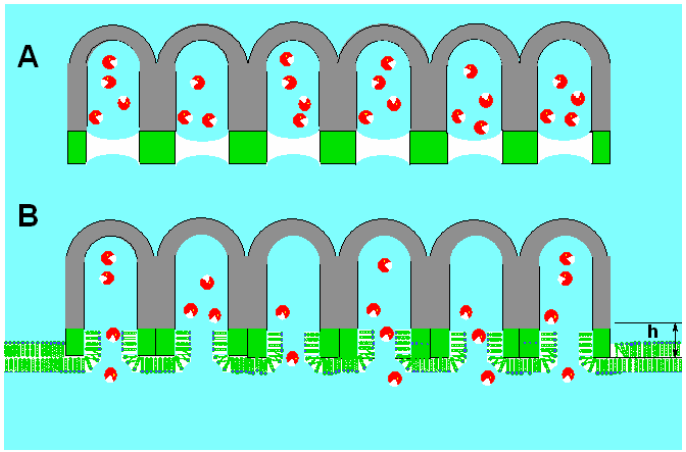

Fig.29. Proposed mechanism of transfection from partially hydrophobic NPM: Hydrophobic modification (green) extends inside only to a depth sufficient for decorating by the 'footprint' lipids and opening the hydrophilic compartments.

D.2.6. Applications of HNPM mediated cell transfection.

Optimized protocols of employing HNPM for cell transfection will be applied in four subprojects, three of which are directed at resolving the problems of intracellular delivery for our collaborators.

D.2.6.1. Intracellular delivery of soluble and poorly soluble drugs. In order to demonstrate the universal nature of the suggested delivery system based on HNPM, we intend to illustrate its traits in optimizing the transfection of representative cancer treatment drugs: doxorubicin (DOX) and paclitaxel (Tx), as the first step towards their application in the more advanced formulation of HNT-based delivery. Having very different solubility in water, they represent a good basis to other HNPM and HNT applications.

DOX--cytotoxic anthracycline antibiotic isolated from *Streptomyces peucetius* var. *caesius* is one of the most commonly used drugs for the treatment of both hematological and solid tumors (reviewed by (Abraham et al., 2005), including human prostate cancer (Bagley et al., 2002), breast cancer (Crown et al., 2002) and urothelial cancer (Calabro and Sternberg, 2002). Paclitaxel (Taxol, Tx) isolated first from the bark of the Pacific yew tree (Taxus brevifolia) is a chemotherapeutic agent with a wide spectrum of antitumor activity. However, its therapeutic applications in cancer therapy are limited, in part, due to its low water solubility. Pharmaceutically acceptable forms of Tx may be obtained using stable polymeric micelles that possess an excellent ability to carry poorly soluble pharmaceuticals and which can serve for targeting of the drugs to the site of disease (Torchilin, 2004).

We anticipate elucidating on optimal formulations (pore dimensions, load, and solvent to load from) for each type of a drug load to identify optimal choices for soluble and poorly soluble substrates. The optimal ones will be applied for other applications of HNPM transfection system and in HNT-based targeted drug delivery. Other questions that are more conveniently addressed with HNPM system, rather than with HNT, will also be studied here. They include: (i) undesired cytotoxicity due to loss of cargo in serum is shown to be insignificant (see **Fig. 123**) but requires investigation in greater details to identify the limitations; (ii) we need to clearly demonstrate the extend of HNPM applications in terms of optimal storage and the shell life time for HNPM, the number of times each membrane can be used and whether the efficiency of transfection depends on the cell type. When compared with other transfection reagents, it should clearly formulate the advantages of HNPM system as: a) **low toxicity** (chemical reagents are eliminated), b) **low cost** (HNPM are reusable), and c) **reliable system for cell transfection**.

D.2.6.2. Intracellular estrogen receptor binding (with J. Arterburn, NMSU). The assessment of estrogen responsiveness is crucial for accurate prognosis and therapy in breast cancer, because proliferation is stimulated in estrogen-responsive tumors. Estrogen receptor positive (ER+) breast tumors are largely responsive to antiestrogen therapy. However, the anti-estrogen, tamoxifen, has also been shown to cause endometrial cancer. These results indicate the complex biology of tumor growth. A new paradigm is emerging with the recent discovery of a new type of estrogen receptor, GPR30, a G-protein coupled receptor that mediates rapid non-genomic cell signaling pathways including generation of secondary messengers Ca^{2+} and NO, as well as activation of receptor tyrosine kinases.(Prossnitz et al., 2006a,b)

Professor Arterburn's research group develops novel ligands targeted for intracellular estrogen receptors. Their synthetic fluorescent estradiol derivatives facilitate the identification of GPR30 as a functional intracellular transmembrane estrogen receptor, (Revankar et al., 2005) and enables biomolecular screening with flow cytometry that identified novel non-steroidal GPR30 selective small molecules (Bologa et al., 2006). The fluorescent probes employed in these studies possess a charged AlexaFluor dye that prevents membrane permeability and necessitates the use of saponin to provide access to the intracellular receptors. Additional recent studies involving a series of synthetic estrogen probes possessing ionic or neutral substituents and metal chelates demonstrated the effect of charge on intracellular concentration (Revankar et al. 2007, Ramesh et al. 2006). Efforts to develop membrane permeable fluorescent estrogen probes have been unsuccessful.

The inability to study intracellular receptor binding in whole cells with charged probes is a major limitation. The development of technology that enables transmembrane delivery of charged/polar fluorescent probes will be a significant advantage for characterizing the intracellular localization of receptor targets. It would also support the development of intracellular competitive binding assays that would facilitate the identification of synthetic ligands and modulators of receptor activation for use as molecular probes and drug discovery to identify new potential therapeutics.

Optimized intracellular delivery based on HNPM will be applied to facilitate screening of otherwise cell membrane impermeable estrogen probes.

D.2.6.3. HIV inhibition by zinc finger proteins (with M.Germann, GSU). Professor Germann's laboratory has generated small zinc finger proteins that target HIV RREIIB RNA with high affinity (Mishra et al. 2007). This is expected to abolish the action of rev protein and, consequently, disrupt the viral propagation. The impeding step in furthering progress of his research is difficulty to test their designed molecules with whole cells. They face a similar impediment in studying HTLV/HIV integrase mediators, that they developed. They anticipate showing these mediators activity in biochemical assays but there is no suitable intracellular vehicle for delivery and cell studies.

We will optimize HNPM and PHNPM (if necessary) delivery protocols for in vitro cell studies and investigate the uptake and subcellular localization of such zinc fingers. The proteins will be labeled for microscopic studies of transfection using AlexaFluor dye.

D.2.6.4. Therapeutic nucleic acids applications: antisense DNA and siRNA vectors (with I. Frolov, UTMB). Therapeutic nucleic acids (TNA) - based therapies can specifically target processes responsible for the growth and survival of cancer cells and thus gained significant interest for cancer treatment in recent years. TNAs include ribozymes, antisense oligonucleotides (AS-ODNs) and small interfering RNAs (siRNAs). Antisense techniques are used to deactivate disease-causing or undesirable genes so that they cannot produce harmful or unwanted proteins. This deactivation can be achieved by introduction TNA into the cell. The antisense segment pairs with the mRNA, preventing the synthesis of protein by the mRNA. Despite a very high in vitro efficiency of TNA-based therapies, their clinical development is burdened by poor specificity and intracellular delivery.

We have already demonstrated the translocation of DNA oligos through the cell membrane, including HeLa and BHK-21 cell lines. Together with Professor Frolov's laboratory, who applies antisense technology for developing vaccines against human and animal pathogens, we will first investigate in detail the application of HNPM delivery approach for TNA transfection in vitro. A current approach in Frolov's laboratory is based on viral particles, which unfortunately are prone to enzymatic response (Agapov et al. 1998; Frolov et al. 1996, 1999; Petrakova et al., 2005, Gorchakov et al., 2007; Volkova et al, 2006). The HNPM based delivery systems offer a promising alternative overcoming the problem of enzymatic complications. Upon finding the optimal formulations, they will be employed in more suitable for clinical studies approach with using targeted HNT as the delivery system (see next chapter).

D.3. Targeted drug delivery based on multifunctional hydrophobic nanotubes.

D.3.1. Rationale and anticipated results.

The current concept of intracellular drug delivery requires endocytosis as a mechanism for transfection. State of the art transfection reagents use in laboratory settings are different from the state of the art drug delivery systems. For the latter, so-called long-circulating immunoliposomes are usually considered first, in which both PEG protecting polymer and the targeting moiety (usually a monoclonal antibody or its fragment) are present on the liposome surface.

We hypothesize that hydrophobic nanotubes (HNT) targeted by ligands for attachment at the surface of specific (*e.g.*, cancerous) cells can be successfully utilized as a universal drug delivery system (DDS). This DDS employs the same hydrophobicity switching mechanism as HNPM but can be formulated in nanometer sized individual tubes that will possess the properties of: (i) **long circulating** without enzymatic degradation, due to PEG surface modification (Klibanov et al., 1990), (ii) **enhanced permeability and retention** (EPR) effect, due to their dimensions (Maeda et al., 2001; Maeda et al., 2000), (iii) **specificity** of cell addressing, due a specific affinity to chosen cells (Torchilin, 1985; Blume et al., 1993; Torchilin et al., 2001), (iv) **low undesired toxicity**, because delivery systems remains intact and is not required to internalize into cells, and v) **universal carrier**, due to their ability to carry and release various cargo without need for endocytosis to transfect into cells and pH-triggereing for dissemination inside the cytoplasm.

The hypothesis will be verified using in vitro studies of transfecting efficiency and specificity for a panel of cancer cell lines with representative small molecule (DOX, Tx) and therapeutic nucleic acids (AS-ODN, siRNA).

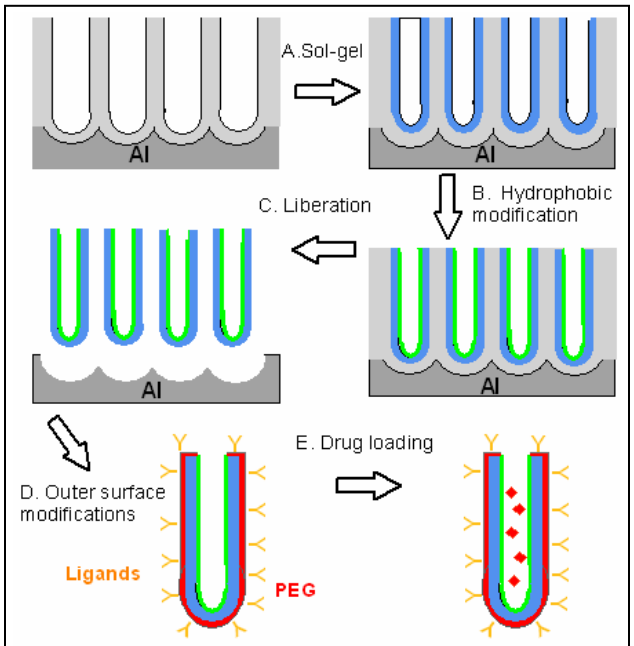

Fig. 30. Fabrication process of HNT-based targeted DDS: Anodized alumina membrane grown on aluminum with desired depth and pore diameter is decorated by silica layer (blue) using sol-gel (**A**); the pores are hydrophobically modified inside (green) (**B**) and, after polishing edges, the nanotubes are freed by dissolving the template (**C**); the outer surface is modified by a mixture of PEG polymer and desired ligands (**D**); drugs (red diamonds) can be loaded at the end (**E**) or before (**C**).

D.3.2. Preparation of HNT for intracellular transfection.

We have demonstrated that silica nanotubes can be conveniently synthesized using sol-gel technique with anodized alumina membranes as templates. Similar method will be applied with small alterations appropriate for certain applications.

D.3.2.1. Preparation of PEGylated HNT. In a typical scenario, the preparation starts with making the alumina template, usually by anodization of aluminum in oxalic acid at 40 V, as explained in D.2.2. We have designed a set up that can be easily scale up, where, instead of foil, a ∅ 2" × 8" long aluminum rod (cylinder) is fastened in a rotoevaporator for anodization and further modification. Rotating the rod provides effective stirring. The rod can be used multiple times without need for the initial anodization, which together with larger surface area and convenient handling, offers a high yield of nanotube. The pores of 50 -90 nm in diameter will be grown to the desired length (300- 3000nm). Immersing this alumina template into the sol-gel precursor (ethanol : tetraethylorthosilicate : 1M HCl at 50:5:1 ratio by volume) and curing would produce a decorated layer of SiO_2 (step A in **Fig. 301**), which, after curing, will make nanotubes with the wall thickness that can be tuned by altering mixture, time or a number of immersions/curing. While still in the membrane, the tubes will be decorated from the inside by a hydrophobic monolayer, as described in D.2.2 (step B). These hydrophobic tubes can be loaded with the substrate, as described in D.2.3, or the loading can be performed at the very last step using the same methods. The top of the alumina membrane template (on the rod) will be gently polished off to remove the top layer of silica in between the tubes. In the standard protocol, the tubes at this point will be

liberated by dissolving alumina template in concentrated phosphoric acid or concentrated NaOH. The remaining aluminum has the pits from the dissolved template left and will not require the preanodization step - a significant expedition for large quantity production. The collected solution of HNT will be neutralized by copious amount of water and filtrated through a 0.2 μm alumina or polycarbonate filter. Finally, the outside surface of collected nanotubes will be either PEG-ylated by plain 2-[methoxy(polyethyleneoxy] trimethoxysilane (Gelest, Inc.), as described in D.2.4., from ethanol solution (2% v/v silane), or modified by a mixed monolayer, as described in the next section D.3.2.2. The zeta –potential, as well as and the dimensions of PEGylated HNT will be verified using Zetasizer Nano-ZS90, as it is important to have a nonzero overall charge for preventing HNT from agglomeration.

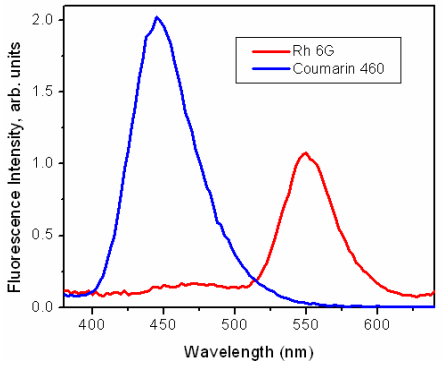

Fig. 31. Fluorescence spectra from doped with coumarin PEGylated HNT and Rh6G dye released from it, as in **Fig. 23**.

In the beginning stages of optimization, when relative contributions of the hydrophobicity switching and the endocytosis mechanisms in drug delivery are compared, it will be useful to discriminate the locations of the drug molecules (*e.g.*, doxorubicin) and of HNT. In that case, HNT will be also fluorescently tagged by dopping sol-gel solution with coumarin dye (10 -100 μM). **Figures 23** and **31** illustrate the significance of fluorescence shift; the spectrum of coumarin is suitable for DAPI filter to be used in fluorescence microscopy measurements.

D.3.2.2. Preparation of targeted HNT. Having HNT 'equipped' with targeting moieties (ligands), such as monoclonal antibodies or their fragments, will allow HNT bind specifically the desired (targeted) cells, *e.g.*, cancerous cells. These ligands should be covalently bound to the outer surface together with PEG polymer, which serves the protecting function. Mixed monolayers on the outer surface of HNT will be prepared using a mixture of PEGylated and aminated silanes in different proportions. The aminogroups will be further employed for binding the desired ligands, such as folic acid, and antibodies for the cell receptors over expressed in cancer cells. Details for chemical modification in this step are outlined in **Fig. 3**. Different lengths of amino group, their proportion to PEG, the type of coupling (maleic anhydride activation + EDC or using gluteraldehide) will be verified to find the optimal protocol. The zeta-potential of HNT and their dimensions will be verified using Zetasizer Nano-ZS90. Prepared this way targeted HNT delivery system is analogous to PEG-protected immunoliposomes, which also have the recognition receptors and PEG layer on the surface, but HNT offers a different mechanism of delivery and a much broader list of substrates for delivery.

We chose three types of receptors for targeting cancer cells: i) folate, ii) EpCAM, and iii) HER2/neu. Biotin receptor will be used as a nondiscriminative receptor for comparison.

Folic acid has emerged as an optimal targeting ligand for selective delivery of attached imaging and therapeutic agents to cancer tissues and sites of inflammation (Low et al, 2004, 2007, Salazar et. al., 2007). The utility of folic acid in these applications has arisen primarily from (1) its ease of conjugation to both therapeutic and diagnosticagents, (2) its high affinity for the folate receptor (K_d ~ 100 pM), even after conjugation to its therapeutic/diagnostic cargo, and (3) the limited distribution of its receptor (FR) in normal tissues, despite its upregulation on both cancer cells (primarily FR-R isoform) and activated macrophages (FR-β isoform). Cancers found to overexpress FR include cancers of the ovary, lung, breast, kidney, brain, endometrium, colon, and hematopoietic cells of myelogenous origin (Salazar et. al., 2007).

EpCAM - (also known as TACSTD1) is epithelial-cell adhesion-molecule found to be frequently over expressed by carcinomas of lung, colorectal, breast, prostate, head and neck, and hepatic origin, and is absent from haematologic cells (Smirnov, 2005). It has been suggested that EpCAM receptor can be used for capturing rare circulating tumor cells (Nagrath, 2007)

HER2/neu – human epidermal growth factor receptor 2, is a cell-surface protein involved in cell development. When activated in cancer cells, HER2 accelerates tumor formation. About 20-30% of breast cancers overexpress HER2.

D.3.2.3. Loading HNT with cargo. The process of loading HNT is very similar to that for HNPM. If the nanotubes are not loaded prior to their liberation from the template, they will be loaded after all modifications either from ethanol solutions directly or from aqueous solution via ethanol induced pores wetting, as described above in D.2.3.

D.3.3. Expected results and significance.

We anticipate identification of optimal diameter and length for dully PEGylation HNT with suppressed endocytosis caused by their elongated shape. This shape will be applied for constructing targeted HNT with folate, HER2/neu, or EpCAm ligands and loaded with doxorubicin or paclitaxel. Evaluating their toxicity for intacellular delivery into the corresponding cell lines should demonstrate the feasibility of targeted HNT as a universal drug delivery system.

D.3.4. Potential pitfalls.

Different cell lines often have different propensity towards endocytisis and it could be that PEGylated nanotubes will have different optimal dimensions to minimze that effect. We will address it by investigating the effect with a panel of cell lines.

The matter of targeted HNT orientation with respect to the cell membrane may appear important. **Figure 1** suggests that the preferred orientation is when the tubes are perpendicular to the membrane. This orientation is naturaly achieved for delivery from HNPM but, for targeted HNT with uniformly distributed ligands on the outer surface, the orientation of tubes towards the membrane is random, at least at the beginning. The tubes should reorient when lipids start decorating their hydrophobic interior but the probability of this happenning can only be verified experimentally. If necessary to improve the odds, a more sophisticated outer surface modification will be performed, in accordance with **Fig. 32**. In this case, the outer surface will not be modified uniformly. Instead, after polishing, the edges will be aminated/passivated, which, after liberation of nanotubes, will allow their different modification based on specific chemistry of amines..It will provide a localized area near the nanopore opening for the final modification with the ligand (step E), while the remaining outside surface will be PEGylated. The resulting HNT-based targeted DDS will have PEG protected most of the outer surface, while the ligands will provide the most desirable nanotube orientation with respect to the cell membrane.

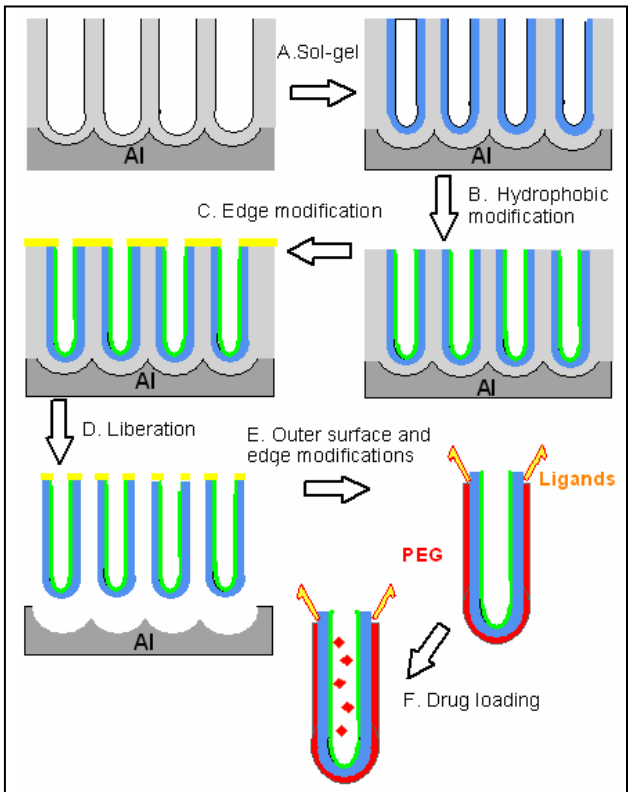

Fig. 32. Fabrication of HNT-based DDS with oriented targeting: Anodized alumina membrane is decorated by silica layer (blue) using sol-gel (**A**); the pores are hydrophobically modified inside (green) (**B**); after polishing, the edges of nanotubes are decorated with aminosilane (APTS) (**C**), HNT are liberated by dissolving the template (**D**); the desired ligands are attached to the aminated edges and the remaining surface is PEGylated (**E**); drugs are loaded in the end (**F**)

As it was mentioned, the described protocol for mass production of HNT can be easily scaled up, which is important for future applications. Nevertheless, there are likely more convenient recepies, which would minimize the overall cost of production. Silica nanotubes provide a convenient platform for initial studies, but we plan to evaluate other (*e.g.*, polymeric) protocols of producing hydrophobic inside nanotubes.

D.4. Main protocols that will be used throughout the study

D.4.1. Interaction of HNPM with cells in vitro.

To assess the efficacy of HNPM transfection into cells, a number of normal- and tumor cell lines will be assessed, including primary diploid fibroblasts, hTERT-RPE1-, HeLa-, and CFPAC-1 cells. These cell lines

vary widely in their ability to be transfected by standard methods, and will therefore represent an important proof-of-concept for the applicability of this technology. Moreover, these cell lines are routinely used in the Shuster lab for cell cycle studies, and require no special considerations in their culture and care. Cells plated onto coverslips at ~70% confluence will be inverted on drug-loaded HNPM and incubated for 1-2 hours at 37^{o}C. After incubation, cells will be briefly washed with PBS three times and mounted onto a temperature-controlled chamber containing phenol red-free media and imaged live. Imaging will be performed using a Zeiss Axiovert200M inverted microscope equipped for standard epi- and structured illumination fluorescence microscopy. Images will be acquired using a Axiocam MrM CCD camera driven by Axiovision 4.5 software.

D.4.2. Interaction of HNT with cells in vitro.

D.4.2.1. Targeted HNT association with cells. To investigate targeted HNT association with attached cells, cells will be cultured and treated over a range of HNT concentrations. Cells will then be washed and imaged as described above. Although our preliminary data with liposomes (**Fig. 18**) suggests that drug delivery can be achieved in the absence of endocytosis, our ability to resolve individual HNT (**Fig. 22**) will allow us to discriminate between true drug delivery and endocytosis.

D.4.2.2. Cytotoxicity test. To determine whether HNT can successfully deliver a cytotoxic dose of proapoptotic drugs, several adherent cancer cell lines (HeLa, CFPAC-1, MCF-7 and BT-483) will be incubated in serial dilutions of different HNT formulations of pro-apoptotic drugs, and plates will be incubated for 5-24 hours at 37^{o}C. After the incubation, the plates will be washed 3 times with Hank's buffer and cell viability will be assayed using MTT Celltiter 96. The cells will be incubated for 3 and 12 h at 37^{o}C, and analyzed by optical absorbance at 490 nm with a 96-well plate reader. As a control, "empty" HNT at similar concentrations will be used as a carrier controls.

OVERALL INNOVATIVE ASPECTS OF THIS PROJECT. We have demonstrated the unique properties of **nanoporous structures** (nanotubes and nanoporous membranes) with hydrophobic interior, that are susceptible to be decorated by the lipids only in close proximity to the cell membrane, where the concentration of lipids is the highest. We propose to utilize this **hydrophobicity switching mechanism** for biological and medicinal applications. Namely, the proposed effect is applied for: (i) **cell transfection from hydrophobic nanoporous membranes** (HNPM) and (ii) **targeted intracellular drug delivery using hydrophobic nanotubes** (HNT). Both are characterized by universal applicability in cell transfection that does not require endocytosis and thus should have low side effects.

OVERALL PROJECT SIGNIFICANCE. If proven effective, this mechanism of intracellular delivery can be applied in treatment of various diseases including cancer and for studying fundamental aspects of cell biology. It can be particularly important by offering the anticipated **low cell toxicity, and high specificity universal approach that was never discussed before**.

FURTHER DIRECTIONS. Currently we have scheduled investigation of cytotoxicity of targeted HNT formulation only for *in vitro* studies on cancer cells. When the results prove to be promising, we intend to seek collaborators, who have the capabilities to do animal studies, such as xenograft on nude mice. We have discussed this option with Professor V. Torchilin (Northeastern University), who expressed interest in pursuing this possibility and confirmed it in his letter.

Other exciting new directions lay in the possibilities of employing HNT in treatment of other cancers and other diseases. Because of the universal approach in delivery, these HNT can be tailored for the task of intracellular delivery by appropriatly chosen drugs and ligands/receptors. The stages of HNT preparation and loading are effectively separated and this opens a broad range of opportunities in elucidating each of them separately. One example of exploring new ligands is pursued by Professor Valery Petrenko (Auburn University), who has developed an extensive set of phage display libraries for various cancer cell lines. He has demonstrated that proteins of these phage probes can be used as highly selective recognition elements, and expressed an interest in using our HNT delivery system in combination with such proteins.

D.5. Timeline, management and data collection

PI will supervise directly all experiments and manage accomplishment of the aims. Protocols of experiments will be discussed with PI and CoPI, reviewed and approved by PI and CoPI. Consultants (Professors J.Arterburn, I.Frolov, and M.Germann) will contribute their samples, review and approve the experiments with them. All data will be documented in notebooks of participants, and after accomplishment of the work will be stored in PI's and CoPI's offices. Original data will be stored in personal computers of the participants and will be backuped. Samples of HNT will be labeled and stored for future references in PI's laboratory.

Timetable for completing this project.

Aim	Year 1	Year 2	Year 3	Year 4
1.1	+++	++	+	
1.2	+++	++	++	
1.3	+	++	+++	++
2.1	+	++	+++	++
2.2	+	++	+++	++
2.3	+	++	+++	+++

D.6. Availability of collected data and materials for research community

We will follow "Principles for recipients of NIH research grants and contracts on obtaining and disseminating biomedical research resources" (http://ott.od.nih.gov/RTguide.html). Briefly, both HNPM and HNT will be distributed to potential users by their request and signing corresponding material transfer agreements. The data will be published in peer reviewed journals and reported on international conferences. To protect legitimate proprietary interests of the New Mexico State University, attract investments of commercial organizations, preserve incentives for commercial development, and timely transfer to industry for commercialization, some data will be patented in parallel with their publication.

E. Human Subjects

Not involved

F. Vertebrate Animals

No experiments with animals are scheduled at this time but we are planning to seek collaborators in the future (when *in vitro* experiments show promising results) who would have the capabilities to conduct research with animal models (human tumor xenografts on mice) and verify our findings *in vivo*.

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