Michael Grossutti, Ryan Seenath and Jacek Lipkowski “Infrared and Fluorescence Spectroscopic Investigations of the Acyl Surface Modification of Hydrogel Beads for the Deposition of a Phospholipid Coating”, *Langmuir*, **2015**,*31*,11598-11604.

Infrared and Fluorescence Spectroscopic Investigations of the Acyl Surface Modification of Hydrogel Beads for the Deposition of a Phospholipid Coating

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Abstract

The scaffolded vesicle has been employed as an alternative means of developing natural model membranes and envisioned as a potential nutraceutical transporter. Furthering the research of the scaffolded vesicle system, a nucleophilic substitution reaction was implemented to form an ester linkage between palmitate and terminal hydroxyl groups of dextran in order to hydrophobically modify the hydrogel scaffold. An average tilt angle of 38° of the hydrophobic palmitate modifying layer on the surface of the hydrogel was determined from dichroic ratios obtained from infrared spectra collected in the attenuated total reflection (ATR) configuration. ATR-IR studies of the DMPC coated acylated hydrogel demonstrated that the hydrocarbon chains of the DMPC coating was similar to those of DMPC bilayers and that the underlying palmitate layer had a negligible effect on the average tilt angle (26°) of the DMPC coating. The

permeability of this acylated hydrogel was investigated with fluorescence spectroscopy and the terbium/ dipicolinic acid assay. The hydrophobic modification on the surface of the hydrogel bead allowed for an efficient deposition of a DMPC layer that served as an impermeable barrier to terbium efflux. About 72% of DMPC coated acylated hydrogel beads showed ideal barrier properties. The remaining 28% were leaking but the half-life of terbium efflux of the DMPC coated acylated hydrogel was increasing and the total amount of leaked terbium was decreasing with the incubation time. The half-life time and the retention were considered a marked improvement relative to past scaffolded vesicle preparations. The process of acylating hydrogel beads for efficient DMPC deposition has been identified as another viable method for controlling the permeability of the scaffolded vesicle.

Introduction

Lipid vesicles have served as useful model biological membrane systems, and have been the focus of efforts to develop artificial biological cells.¹⁻⁴ Their biocompatibility, modifiable surface properties, and enclosed internal volume **have** inspired potential applications in therapeutic drug delivery.⁵⁻⁷ However, lipid vesicles have poor mechanical stability, which can limit their biotechnological applications.¹ Their small size makes physical manipulation difficult, and can make them inaccessible to characterization by electrochemical and surface analytical techniques.^{7,8} Supported vesicle systems can provide solutions to these challenges. In a previous study, large (~100 μm diameter) hydrogel beads (Sephadex™) were used as a scaffold to provide structural support for a 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and cholesterol bilayer.⁹ The large scaffold is a design feature that allows for their characterization by attenuated

total reflection infrared spectroscopy (ATR-IR), and is intended to allow for their study with fluorescence spectroscopy, and electrophysiological patch-clamp.

Our previous work established the ability to use polarized ATR-IR spectroscopy to make quantitative molecular orientation measurements on scaffolded vesicles.⁹ However, permeability studies revealed a leaky membrane coating.⁹ The present work addresses a way to improve barrier properties in order to extend our characterization of the scaffolded vesicles to the patch-clamp technique. To improve the membrane barrier properties of the scaffolded vesicles, the hydrogel support was functionalized with hydrophobic acyl chains. This approach has been successfully used in the literature to prepare impermeable lipid membranes supported on polymeric microgel beads.¹⁰ To achieve a bilayer coating, the modified hydrogel scaffolds were subsequently incubated in phospholipid vesicles.

The acylation reaction is expected to significantly alter the infrared spectrum of the hydrogel beads. The attachment of acyl chains via an ester linkage should result in the appearance of several characteristic spectral features. The C-H stretching region ($3050\text{--}2750\text{ cm}^{-1}$) should display increased intensity and its appearance should be significantly altered as a result of the acyl chains. The ester linkage should give rise to a distinct carbonyl band in the C=O stretching region ($1850\text{--}1650\text{ cm}^{-1}$). Furthermore, ester linkage C-O stretching vibrations are expected to alter the appearance of the infrared fingerprint region. An examination of these defining spectral features was performed using ATR-IR spectroscopy in order to confirm the success of the acylation reaction.

In this study, the scaffolded vesicles were investigated using fluorescence microscopy, fluorescence and ATR-IR spectroscopies, and electrophysiological patch-clamp. Fluorescence microscopy was employed to visualize the membrane coating by use of fluorescent-tagged

phospholipids, and to examine the membrane permeability by utilization of a fluorescent probe. Fluorescence spectroscopy was used to further assess the permeability of the lipid coated hydrogel bead and to relate its permeability to past scaffolded vesicle preparations. ATR-IR spectroscopy was used to examine the molecular orientation of the phospholipid chains. This necessitated the use of deuterium substituted acyl chains, in order to avoid interfering bands. The novelty of this study lies in the design of a model membrane supported on sephadex beads with improved barrier properties. These scaffolded vesicles have potential to be used in a tandem ATR-IR/patch-clamp experiment to study potential controlled membrane processes and for drugs or nutraceuticals encapsulation.

Experimental

Chemicals and Solutions

Lipid stock solutions were prepared from powdered 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (Avanti Polar Lipids, Alabaster, AL) and 1,2-dimyristoyl-*d*54-*sn*-glycero-3-phosphocholine (DMPC-*d*54) by dissolving in chloroform (Sigma Aldrich, Mississauga, ON). 1-Myrisoyl-2-[12-[7-nitro-2-1,3-benzoxadiazol-4-yl)amino[hexanoyl]-*sn*-glycero-3-phosphocholine (NBD-DMPC) (Avanti Polar Lipids, Alabaster, AL) was purchased in chloroform. All aqueous solutions were prepared with Milli-Q (EMD Millipore Canada, Etobicoke, ON) water. Stock terbium (III) chloride and dipicolinic acid (Sigma Aldrich, Mississauga, ON) solutions, both with final concentrations of 1.0 mM were prepared for the fluorescence spectroscopy measurements.

Sample Preparation

Hydrophobically Modified Sephadex G10

Sephadex G10 (Sigma Aldrich, Mississauga, ON) beads were incubated in 1 M palmitoyl chloride (Sigma Aldrich) in dichloromethane for ~8 hr at room temperature, under gentle stirring. Upon reaction completion the beads were allowed to settle and the organic solution removed. The hydrogel beads were then rinsed repeatedly with methanol and allowed to dry. For the fluorescence spectroscopy studies, the modified hydrogel beads were incubated in a 1.0 mM aqueous solution of TbCl₃ for a minimum of 48 hours. Dry modified hydrogel beads were characterized with ATR-IR spectroscopy. The distribution of hydrated particles size is shown in Figure 1S of Supporting Information. The average size of hydrated hydrogel beads was $107 \pm 20 \mu\text{m}$.

Phospholipid Vesicle Preparation

Dry lipid films were prepared by combining DMPC and NBD-DMPC chloroform solutions in a test tube, at a 99:1 molar ratio. The chloroform solvent was slowly evaporated under a stream of argon while vortexing. After evaporation the test tubes were stored in a dessicator under vacuum for at least 24 h prior to use. For ATR-IR experiments, DMPC-*d*₅₄ was used in the place of DMPC. To make vesicles, a volume of 0.15 M NaCl solution was added to the dry lipid film such that a ~5 mg/mL solution was obtained. The mixture was sonicated for ~5 min at ~45 °C.

Scaffolded Vesicle Preparation

To prepare scaffolded vesicles, phospholipid vesicles and hydrophobically modified Sephadex G10 hydrated in 0.15 M NaCl were incubated at ~45 °C for ~1 hr with constant

vortexing. The system was then allowed to incubate further at room temperature for ~24 hr. The scaffolded vesicles were then rinsed thoroughly with 0.15 M NaCl prior to use.

Fluorescence Microscopy

Fluorescence microscopy experiments were performed on an Olympus BX51 microscope equipped with a QImaging Retiga 2000R camera. An Olympus U-RFL-T mercury burner served as the light source. To obtain images, fluorescent scaffolded vesicles were dropped onto a microscope slide and allowed to settle. For permeability experiments, Sephadex G10 or scaffolded vesicles were incubated in $\sim 10^{-5}$ M sulforhodamine B on the microscope slide and images were obtained.

Fluorescence Studies of Bead Leakage

The acylated hydrogel was incubated in a 1.0 mM TbCl_3 solution for 48 hours and a 10 μL aliquot was deposited into a quartz cuvette containing 3.00 mL of a 0.125 mM dipicolinic acid (DPA) solution. The ratio of desiccated beads in 1.0 mM TbCl_3 solution was 10% w/v.

As the Tb^{3+} leaked from the acylated hydrogel and complexed with the antenna ligand (DPA) in the supernatant, the fluorescence emission intensity was amplified upon irradiation with an excitation wavelength of 270 nm. The fluorescence intensity of the amplified 585 nm band of terbium was monitored over 200 min with a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian Inc., Mississauga, ON). A calibration curve was measured to relate the concentration of the Tb^{3+} /DPA complex to fluorescence intensity. This was performed without the hydrogel beads. The fluorescence intensity measurements were repeated with the Tb^{3+} containing acylated hydrogel coated with a DMPC bilayer over the span of 27 days which

produced a set of kinetics curves that were used to assess any improvements to the DMPC coating. The Tb³⁺/DPA complex provided relatively narrow and intense fluorescence bands giving a strong signal with a decreased quantity of the sample. The fluorescence intensity as a function of Tb³⁺ to DPA stoichiometry and complex concentration has been extensively characterized.⁹ An ionic analyte was required due to the relatively low permeability of phospholipid membranes to charged species and it is small in size compared to organic chromophores.

For the comparison of the kinetics plots, the fluorescent intensities were calibrated with respect to the concentration of terbium in the supernatant solution above the sedimented scaffolded vesicle preparation. A fluorescence intensity to concentration calibration was necessary since the previously implemented method of disrupting the DMPC coating with the surfactant Triton X-100 was deemed inappropriate for determining the retention of terbium by the coated acylated hydrogel.⁹ The maximum intensities obtained for the kinetics plot of the uncoated acylated hydrogel were then normalized with respect to that of an uncoated non-acylated hydrogel. Furthermore, the maximum intensities of the kinetics plots of the coated acylated hydrogel were then normalized with respect to the uncoated acylated hydrogel. This normalization procedure allowed for the terbium retention of the coated acylated hydrogel to be quantified along with the rate constant of terbium efflux. The fluorescence kinetics plots were averages of at least three independent measurements. The standard deviation of these measurements was plotted as the error bar.

ATR-IR Spectroscopy

Polarized ATR-IR spectra were collected on a Varian 660-IR spectrometer equipped with an MCT-A detector, a Pike MIRacle ATR accessory (Pike Technologies, Madison, WI), a ZnSe wire-grid polarizer (Pike Technologies, Madison, WI) and a germanium ATR crystal. Spectra were collected at an incident angle of 45° at a resolution of 4 cm^{-1} . When spectra are recorded with a resolution 4 cm^{-1} the data points are computed every 2 cm^{-1} . Since the bands are plotted by interpolation between these points the band center position can be determined with uncertainty 0.5 cm^{-1} .¹¹ A pressure clamp equipped with a concave tip was used to achieve good contact between the sample and ATR crystal. To obtain a spectrum, typically 5000 scans were collected and averaged. Water was used as the background for hydrated systems, and air was used as the background for dry systems.

The spectra are presented in absorbance units defined as $A = -\log(I/I_0)$ where I and I_0 are the single beam reflected intensities for the sample and background, respectively. Absorbance spectra for both p- and s-polarizations (A_p and A_s) were obtained. The absorbance spectra were baseline-corrected, typically with a quadratic function, before further analysis. For quantitative analysis, the dichroic ratio of an absorption band was found by taking the ratio of the p- and s-polarized absorbance spectra ($R = A_p/A_s$) at the band maximum. For quantitative calculations of molecular orientation, the following refractive indices were used: germanium 4.0, DMPC- d_{54} acyl chains 1.42, and water 1.33.^{12,13} The reported scaffolded vesicle spectra were averages of three independent experiments.

Results and Discussion

Hydrophobically Modified Hydrogel Scaffold

The hydrophobically modified hydrogel scaffolds were prepared in a fashion similar to that used by Jin *et. al.*¹⁰ The hydrophobic modification was an acylation reaction, where an acyl chloride underwent a nucleophilic substitution with the hydroxyl groups of the hydrogel. The result is an ester linkage that connected the palmitoyl acyl chains to the cross-linked dextran network of the hydrogel. Since the hydrogel was not observed to swell in organic solvents, the reaction was considered to be limited to the surface hydroxyl groups of the hydrogel beads.

Dried modified scaffolds were characterized by ATR-IR spectroscopy, a surface sensitive IR technique. An infrared evanescent wave penetrated a short distance ($>1\ \mu\text{m}$) into the sample, and decayed exponentially with distance from the sample-ATR crystal interface. As a result, the surface region of the modified hydrogels contributed most strongly to the IR spectrum. A comparison of the spectral features of dried unmodified (non-acylated) and hydrophobically modified (acylated) hydrogel follows.

Figure 1 shows the C-H stretching, carbonyl stretching (Fig. 1a), and fingerprint (Fig. 1b) regions of the IR spectra for acylated and non-acylated hydrogel. While only the p-polarized spectra are shown, the s-polarized spectra displayed the same behaviour. The C-H stretching region of the acylated hydrogel differed substantially from that of the non-acylated hydrogel. The C-H stretching region of the non-acylated hydrogel had spectral contributions from glucose C-H and CH_2 groups as well as C-H and CH_2 groups associated with the epichlorohydrin cross-linking agent. Upon acylation, the acylated hydrogel spectra showed distinct $\nu_s(\text{CH}_2)$ and $\nu_{as}(\text{CH}_2)$ bands at 2850 and $2921\ \text{cm}^{-1}$, respectively. The dominance of these bands (compared to the non-acylated hydrogel spectrum) was attributed to the added contributions from the hydrocarbon chains of the ester linked palmitoyl groups. The prominent shoulders observed at 2870 and $2954\ \text{cm}^{-1}$, were assigned to $\nu_s(\text{CH}_3)$ and $\nu_{as}(\text{CH}_3)$, respectively. The appearance of

these bands is attributed to the terminal methyl groups of the palmitoyl chains. Therefore, the C-H stretching region was consistent with a successful acylation of the hydrogel scaffold.

Figure 1 – ATR-IR spectra of acylated (black) and non-acylated hydrogels (red); a) the C-H stretching region (3050-2750 cm⁻¹) and carbonyl stretching region (1850-1650 cm⁻¹), b) the fingerprint region.

The $\nu(\text{C=O})$ band was observed at 1740 cm⁻¹ in the acylated hydrogel spectrum shown in Fig 1a. Bands in this region were not observed for the non-acylated hydrogel. The emergence of this carbonyl band was strong evidence of an ester linkage between the palmitoyl chains and the hydroxyl group of the hydrogel. Further evidence of an ester linkage was present in the complex fingerprint region of the ATR-IR spectrum. Many overlapping bands were present in the fingerprint region, making analysis difficult. However, there were clear differences in appearance between the acylated and non-acylated hydrogel spectra in the fingerprint region. The most prominent changes were the emergence of a shoulder at ~1170 cm⁻¹ and a shifted peak

at $\sim 1110\text{ cm}^{-1}$. These features arose from the ester C-O stretching vibrations, which were known to have two (or more) bands in the fingerprint region between $1300\text{-}1000\text{ cm}^{-1}$.¹⁴ The higher wavenumber band was expected to arise from the C-O stretch between the sp^2 hybridized carbonyl carbon and ester linkage oxygen.¹⁴ The lower wavenumber band was expected to result from the C-O stretch between the sp^3 hybridized hydrogel network carbon and ester linkage oxygen.¹⁴ Taken together, the ATR-IR spectra provided strong evidence of a successful hydrophobic modification of the hydrogel beads through the attachment of palmitoyl acyl chains via an ester linkage.

When the dried acylated hydrogel beads were added to water, they were observed to float and aggregate. When allowed to swell for $\sim 24\text{ hr}$, many beads were observed to sediment and continued to remain aggregated. In contrast, unmodified hydrogel beads did not aggregate in the aqueous environment and immediately sedimented. This behaviour was further evidence of a successful hydrophobic surface modification of the hydrogel beads. When the acylated hydrogel beads were incubated in a phospholipid vesicle solution, their tendency to aggregate was no longer observed, which implied a hydrophilic surface character. This observation strongly suggested the presence of a phospholipid coating on the acylated hydrogel. **Figure S2 of Supporting Information shows pictures of aggregated acylated hydrophobic hydrogel beads and non-aggregated beads after addition of DMPC vesicles.**

ATR-IR Spectroscopy of Scaffolded Vesicles

DMPC- d_{54} Chains

The molecular order and orientation of the DMPC acyl chains was studied using ATR-IR spectroscopy. In order to avoid spectral interference from the hydrogel scaffold and palmitoyl

chains, DMPC with deuterated acyl chains (DMPC- d_{54}) was used for the ATR-IR studies. DMPC- d_{54} displayed C-D stretching vibrations located between 2250 and 2000 cm^{-1} . Of interest in this work was the $\nu_s(\text{CD}_2)$ and $\nu_{as}(\text{CD}_2)$ bands positioned at $\sim 2090 \text{ cm}^{-1}$ and 2195 cm^{-1} , respectively. These were the two most prominent bands in the C-D stretching region, and they contained information regarding the molecular conformation and orientation of the DMPC- d_{54} acyl chains.

Figure 2 shows the p- and s-polarized spectra for the C-D stretching region. The peak maxima of the $\nu_s(\text{CD}_2)$ and $\nu_{as}(\text{CD}_2)$ bands were well defined, and were observed at 2090 cm^{-1} and 2195 cm^{-1} respectively. For all-trans DMPC- d_{54} acyl chains, the $\nu_s(\text{CD}_2)$ and $\nu_{as}(\text{CD}_2)$ bands were observed at 2088 and 2191 cm^{-1} , respectively.¹⁵ The higher frequencies observed in this work suggested that the DMPC- d_{54} acyl chains were partially melted and contained a mix of trans and gauche conformers. This was expected for a DMPC- d_{54} bilayer at room temperature.

Figure 2 – P-polarized (black) and s-polarized (red) ATR-IR spectra of the C-D stretching region. The well-defined peaks positioned at ~ 2090 and $\sim 2195 \text{ cm}^{-1}$ belonged to the $\nu_s(\text{CD}_2)$ and $\nu_{as}(\text{CD}_2)$ vibrations. The position and intensity of these bands contained molecular order and orientation information for the DMPC- d_{54} acyl chains.

The determination of quantitative molecular orientation data for scaffolded vesicles using polarized ATR-IR spectroscopy was examined in a previous paper.⁹ This research established suitable experimental parameters for quantitative orientation studies. The present work used a germanium ATR crystal and a 45° incident angle for the study of the scaffolded vesicles, in accordance with the previous.⁹ The theory associated with the determination of quantitative molecular orientation information from polarized ATR-IR spectroscopy was described in detail in reference 9 and other sources.^{9,10,15,17}

The dichroic ratios for the $\nu_s(\text{CD}_2)$ and $\nu_{as}(\text{CD}_2)$ bands were 1.05 and 1.06, respectively. The dichroic ratio for each band was used to find the average tilt angle of the transition dipole moment vectors associated with each of the symmetric ($\theta_s(\text{CD}_2)$) and asymmetric ($\theta_{as}(\text{CD}_2)$) stretching vibrations. The $\theta_s(\text{CD}_2)$ and $\theta_{as}(\text{CD}_2)$ were each found to be $(72 \pm 3)^\circ$ with respect to the surface normal, and were calculated as described in reference 9.

The transition dipole moments of the symmetric and asymmetric CD_2 stretching vibrations were perpendicular to one another. Both of these transition dipole moments were also perpendicular to the vector defined by a trans segment of the DMPC- d_{54} acyl chains. Since the vectors associated with the two transition dipole moments and an acyl chain trans segment were mutually orthogonal, the following trigonometric relationship was used to find the average tilt angle of the acyl chain trans segment (θ_{chain}), with respect to the surface normal.¹⁸

$$\cos^2\theta_s(\text{CD}_2) + \cos^2\theta_{as}(\text{CD}_2) + \cos^2\theta_{\text{chain}} = 1 \quad \text{Eq. 1}$$

The average θ_{chain} was found to be $(26 \pm 3)^\circ$. This was in agreement with previous polarized ATR-IR studies on DMPC bilayer membranes, which had reported tilt angles $\sim 25^\circ$.¹⁹⁻²¹

Furthermore, an average θ_{chain} of 26° ruled out an adsorbed vesicle coating, which would have presented a tilt angle of 55° (isotropic orientation).

The agreement between $\theta_s(\text{CD}_2)$ and $\theta_{\text{as}}(\text{CD}_2)$ also implied that there was no preferential twisting of the DMPC- d_{54} acyl chains. The twist angle (θ_{twist}) of the trans segment of an acyl chain was defined as the angle between the -C-C-C- plane and that formed by the chain axis and surface normal.²² The twist angle was calculated using the following trigonometric relationship.²²

$$\tan \theta_{\text{twist}} = \frac{\cos \theta_{\text{as}}(\text{CD}_2)}{\cos \theta_s(\text{CD}_2)} \quad \text{Eq.2}$$

A twist angle of 45° was calculated for the DMPC- d_{54} acyl chains, which was equivalent to the trivial twist (i.e. the angle at which there was no preferential chain twisting).

Palmitoyl Chains

The C-H stretching region was located between 3000 and 2800 cm^{-1} and predominantly contained information about the ester linked palmitoyl chains. Figure 3 shows this spectral region for the scaffolded vesicles. The symmetric and asymmetric CH_2 vibrations gave rise to the prominent peaks positioned at 2854 and 2923 cm^{-1} , respectively. These frequencies suggested the presence of both trans and gauche conformers in the palmitoyl chains. This was consistent with the molecular ordering observed for the DMPC acyl chains. The dichroic ratios for the symmetric and asymmetric bands were 1.38 and 1.40 , respectively. From these dichroic ratios and using the same relationship as for the DMPC chains previously outlined in equation 1, an average palmitoyl chain tilt angle of 38° was obtained. This suggested that the acylation reaction primarily occurred near the outer surface of the beads. If palmitoyl chains had been covalently linked to hydroxyl groups throughout the scaffold interior, it was expected that an isotropic chain

orientation of 55° would be measured. A palmitoyl chain tilt angle of 38° implied that the acyl linkages were predominantly present at the surface of the hydrogel.

Figure 3 – P-polarized (black) and s-polarized (red) ATR-IR spectra of the C-H stretching region. The well-defined peaks positioned at ~ 2923 and ~ 2854 cm^{-1} were assigned to the $\nu_{\text{as}}(\text{CH}_2)$ and $\nu_{\text{s}}(\text{CH}_2)$ vibrations.

Fluorescence Microscopy of Scaffolded Vesicles in Aqueous Solution

Fluorescent scaffolded vesicles were prepared by incubating the hydrophobically modified hydrogel scaffolds in DMPC:NBD-DMPC = 99:1 molar ratio vesicles. The NBD-DMPC fluoresces green, and serves as a fluorescent probe for the visualization of the scaffolded vesicle membrane. Figure 4a shows an image of a fluorescent scaffolded vesicle. The coronal appearance of the fluorescence suggests that the phospholipids are confined to the outer surface of the scaffold, rather than having been absorbed into the hydrogel matrix. However, the image does not distinguish between a bilayer and adsorbed vesicle coating structure. The ATR-IR experimental results will address this question.

Figure 4 – Fluorescence images. a) Scaffolded vesicle with 1 mol% NBD-DMPC, b) Hydrogel bead incubated in sulforhodamine B (SRB) for ~30 min., c) Scaffolded vesicle incubated in SRB for ~30 min, showing the exclusion of the SRB probe.

The permeability of the scaffolded vesicle membrane was examined using sulforhodamine B (SRB). SRB is a membrane impermeable red fluorescent probe. Figures 4b and 4c show fluorescence images of a hydrogel bead and a scaffolded vesicle incubated in SRB for 30 minutes, respectively. As is evident from Fig. 4b, the SRB accumulated in the hydrogel matrix, which resulted in strong fluorescence intensity across the diameter of the bead. In contrast, Fig. 4c shows that the SRB was excluded from the hydrogel scaffold interior, and therefore did not diffuse through the scaffolded vesicle membrane. This suggested that the scaffolded vesicle membrane was of high quality. These permeability results were a significant improvement upon the previous class of scaffolded vesicles described in reference 9. Therefore, the present scaffolded vesicles were selected for further characterization by fluorescence spectroscopy, and ATR-IR spectroscopy. A discussion of these studies follows.

Fluorescence Spectroscopy of Scaffolded Vesicles in Aqueous Solution

When the desiccated acylated hydrogel beads were placed in water, swelling was observed and the beads remained suspended in solution. This implied that the beads did internalize water even in the presence of the exterior hydrophobic acyl chains. The observation

that the acylated hydrogel swelled when placed in water allowed for the kinetics of terbium efflux from the scaffolded vesicle to be investigated. The fluorescence intensity normalized with respect to that of the uncoated non-acylated hydrogel was obtained in order to assess terbium retention of the coated hydrogels. Figure 5 shows that the retention of the DMPC coated acylated hydrogel was markedly improved with respect the uncoated acylated hydrogel sample. The average retention of the coated acylated hydrogel samples incubated for 27 days was $72 \pm 2\%$ relative to the non-acylated hydrogels, respectively. These values of terbium retention were a significant improvement to the impermeability compared to previous scaffolded vesicle preparations.

Figure 5 – The fluorescence versus leakage time curves; dashed line uncoated hydrogel beads; (○) acylated hydrogel beads and (●) the acylated hydrogel beads incubated for 27 days in DMPC vesicles solution.

The normalized kinetic plot of uncoated acylated hydrogel demonstrated that there was approximately 20 % retention of terbium in the absence of a DMPC bilayer coating (Figure 5). This was expected since the hydrophobic coating should have retarded the efflux of terbium ions. The terbium retention (72 %) of the DMPC coated acylated hydrogel implied a relatively high impermeability. It was evident that the DMPC coating was the main **barrier** of terbium release. The approximately 28 % release of terbium from the 27 day incubated scaffolded vesicle

preparation was most likely due to hydrogel beads that were not fully acylated and therefore, had a lower extent of DMPC coverage. This is confirmed by Figure 6a that shows normalized fluorescence plots recorded for samples incubated with DMPC vesicles at different incubation times.

Figure 6 – a) Normalized fluorescence intensity kinetics data of terbium efflux from the acylated hydrogel coated with a DMPC bilayer. b) Half life times obtained from the kinetics data indicated an improvement in the permeability of the acylated hydrogel that was allowed to incubate in an 0.1 mM Tb³⁺_(aq) solution of DMPC vesicles for: 1 day (●), 6 days (●), 15 days (●), 22 days (●) and 27 days (●).

The results show that the impermeability increased with the incubation time. The retention of terbium increased from ~67% after 1 day to ~72% after 27 days of incubation. In addition the permeability of the leaking DMPC coated acylated hydrogels was reduced. This is shown in Figure 6b that plots the half-life time ($t_{1/2}$) of terbium efflux. For the hydrogels that were leaking, the half-life of the terbium efflux after one day of incubation with DMPC vesicles was measured to be approximately (17 ± 2) min. The half-life was observed to increase to a value of approximately (42 ± 3) min after 27 days of incubation. These results demonstrate that the extent of the DMPC coating was improved over the 27 day interval of incubation. They indicate that

about 70% of acylated hydrogels are coated by DMPC quickly and the remaining 30 % slowly, and suggest that improved acylation or fractionation of acylated hydrogels could give scaffolded vesicles with excellent barrier properties.

Conclusions

ATR-IR spectroscopic studies and qualitative observations support a successful hydrophobic modification of the hydrogel bead scaffold. Quantitative polarized ATR-IR studies performed on the scaffolded vesicles suggested a bilayer coating structure in which the inner leaflet consists of palmitoyl chains and outer leaflet of a monolayer of DMPC molecules. Fluorescence microscopy and fluorescence spectroscopic permeability studies showed that scaffolded vesicles have a durable, low permeability membrane coating. The permeability of the lipid coating decreased with the time in which the hydrogel was incubated with lipid vesicles indicating that the permeability of the system may be controlled by altering incubation time.

It has been demonstrated that the scaffolded vesicle is amenable to spectroscopic studies and represents an interesting system in which the structure and permeability of a model membranes may be investigated. Future research on scaffolded vesicles will focus on a further assessment of their permeability. For example, an investigation into the extent of hydrogel acylation would also provide an additional means of controlling the amount of lipid coating and thus, the permeability of the scaffolded system. This ability to control permeability would be beneficial in nutraceutical carrier applications where temporal dependent retention may be desirable. In addition, this system could be extended to combined spectroscopic and electrophysiological patch-clamping studies which can provide molecular level information about the voltage dependent changes in membrane structure and function.

Acknowledgement

This work was supported by a Discovery Grant from Natural Sciences and Engineering Council of Canada.

ASSOCIATED CONTENT

Supporting Information. Plot of the hydrated particles size distribution and pictures showing aggregation of the hydrophobic acylated hydrogel beads before addition of DMPC vesicles and sedimented beads after addition of DMPC vesicles are shown in the Supporting Information.

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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