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Abstract

Recently, styrene-maleic acid copolymer lipid nanodiscs have become an increasingly popular tool for the study of membrane proteins. In the work we report here, we have developed a novel method for the efficient preparation of labeled nanodiscs, under chemically mild conditions, by modification of the hydrolyzed styrene-maleic acid copolymer. This protocol is designed to be easily accessible to biochemistry laboratories. We use this procedure to prepare various fluorescent nanodiscs labeled with different fluorophores. By studying the development of Förster resonance energy transfer, we demonstrate the rapid exchange of co-polymer between nanodiscs. This demonstration, in conjunction of previous work, indicates that the lipid nanodiscs prepared using this polymer are very dynamic structures with rapid exchange of the different components.

Keywords: SMALP, Membrane Protein, Nanodisc

1. Introduction

The *in vitro* study of membrane proteins can be particularly challenging [1]. This challenge arises from various steps in a typical protocol, that includes over-expression, membrane isolation, solubilization and purification. During solubilization detergents are typically used to extract the proteins from their native lipid environment, and to replace the lipids with detergent molecules. Finding an appropriate detergent is complex, and the delipidation can often result in loss of activity or denaturation [2]. The difficulties associated with using detergents have led to the development of several alternative strategies for maintaining membrane proteins in solution including

amphipols [3], protein stabilized lipid nanodiscs [4], and polymer solubilized lipid nanoparticles [5].

Recently the direct solubilization of membranes using the polymeric detergent styrene maleic acid (SMA) copolymer has become increasingly popular as an alternative to low molecular weight detergent based strategies [6][7][8]. To date, this amphipathic polymer has been shown to solubilize membranes to give nanometric discs-shaped particles. These particles are known as styrene maleic acid lipid particles (SMALP). When used on biological membranes, these particles contain a mixture of protein, membrane lipids and the SMA polymer. These nano-particles are believed to be organized as a protein surrounded by native membrane lipids, the whole maintained in aqueous solution thanks to a belt of the amphipathic polymer [5]. During solubilization, the integral membrane proteins are thus extracted into a nanodisc along with their native lipidic environment. SMA copolymer nanodiscs are compatible with conventional purification approaches, such as affinity chromatography or gel filtration, allowing purification of the SMALP embedded protein [9]. A particularly interesting point is the "almost native" environment, because membrane proteins may need specific lipids to modulate their structure and exercise their function [10][11].

SMA have been already used to solubilize membranes from various organisms such as Gram positive [6] or Gram negative bacteria [12], yeast [8][13], insect and human cultured cells [14][8]. Solubilized proteins exhibit various transmembrane segments number and are indistinctly trapped in SMALPs with a average diameter around 13 nm [15]. Subsequently particles were characterized by various techniques such as transmission electron microscopy [6], dynamic light scattering [6], fluorescence spectroscopy [6][14][7], NMR spectroscopy [16], fourier transform infra red spectroscopy [16], small angle neutron scattering [16] and EPR spectroscopy [17]. SMA purified proteins have also been used to resolve the structure of a bacteriorhodopsin by X-ray crystallography without any conventional detergent being used [18].

For various protocols it could be useful to have chemically modified SMA. For example, for lipid nanodisc immobilization a specific label would be useful. Indeed, there are several articles in which labeled amphipols have been used to immobilize membrane proteins, using a variety of chemistries: oligohistidine tags [19]; biotin tags [20]; and DNA oligonucleotides [21]. Labeled SMA could thus be used for similar experiments while maintaining the membrane proteins in a lipid environment. Fluorescent labeling also provides interesting possibilities for studying membrane protein interactions without

protein modification, for example using fluorescence correlation spectroscopy or energy transfer measurements.

A previously published method to produce labeled SMALPs is based on the functionalization of styrene-maleic anhydride copolymer, and the subsequent hydrolysis of the maleic anhydride to maleic acid under alkaline conditions [22]. This protocol exploits the reactivity of anhydrides towards alcohols and amines that has already been used to directly conjugate SMA with fluorophores, drugs and small molecules[23][24][25]. However, the use of this protocol is limited by the strong alkaline conditions and the use of non-aqueous solvents not frequently found in biology laboratories.

Here, we propose a novel protocol for modification of SMA in aqueous solution, that can be used for making labeled nanodiscs. This method is based on the reaction coupling of carboxylic acids to primary amines using a carbodiimide. This reaction is frequently used for protein modification [26]. Unlike the protocol based on modification of the maleic anhydride form, our protocol maintains chemically mild conditions compatible with many biomolecules throughout the procedure. This makes the chemistry easily accessible to biological laboratories, and will allow a wider range of molecules to be attached to the SMA. We have used our protocol to prepare fluorescent SMALPs, and used these to examine the exchange of polymer between lipid-nanodiscs.

2. Materials and Methods

2.1. SMA-SH preparation

Hydrolyzed styrene-maleic acid copolymer (SMA) with a 3:1 styrene to maleic acid ratio in NaOH solution at 25% *w/v* was provided by Polyscope (Xiran SL25010 S25). All modification steps were performed at room temperature with stirring. Typically, 1% of carboxyl groups were targeted for modification, based on the number of carboxylate groups present in the polymer composition this is 4.47 mmol/g. To a polymer solution at a concentration of 25% an appropriate amount of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma, St Louis, USA) was added for the degree of labeling targeted. Thus, for modification of 1% of the carboxyl groups in 1g of polymer 45μmole of EDC was added. To stabilize intermediates, N-hydroxysulfosuccinimide (Sulfo-NHS) was added at a 20% molar excess (54 μmole / g polymer). Both, EDC and Sulfo-NHS were dissolved in MES 75mM pH 5.8 in 25% ethanol. They were then added to the polymer and incubated 20

minutes at room temperature with stirring, before a 2 fold excess (108 μmole / g polymer) of cystamine dihydrochloride (Sigma, St Louis, USA) dissolved in 25mM MES pH 7.0 25% EtOH was added slowly with mixing. When the solution became less translucent, EtOH 96% was added drop-wise until the solution clarified. The reaction was then allowed to proceed for 12h at room temperature with stirring, and covered to avoid EtOH evaporation.

The modified polymer was then separated from reagents and low molecular weight products by dialysis for 2 hours at room temperature against 1 liter Tris 20 mM pH 8.0 with a 8000 MW cut-off membrane (Spectra/Por membrane, Spectrum Laboratories), the buffer was changed once during dialysis. SMA-SH solutions were stored at 4°C until use.

2.2. SMA-SH Labeling

Immediately prior to use the modified SMA was reduced by addition of a five fold excess (0.27 μmole / g polymer) of DTT and dialyzed 2 hours at room temperature against 10 volumes of Tris-HCl 20 mM pH 8.0.

Reduced and dialyzed SMA-SH were labeled with Atto488- or Atto532-maleimide according to Atto-TEC recommendations [27]. Briefly, 1 mg of maleimide was dissolved in 200 μL on DMSO, and then a 1.3 fold excess of this solution was added to the SMA-SH solution and incubated for 24 hours at 22°C in Tris-HCl 20 mM pH 8.0 NaCl 200 mM.

After the reaction, unreacted maleimides were removed with MicroBioSpin Chromatography Columns (Promega), equilibrated with Tris 20 mM pH 8.0, NaCl 200 mM, according to product recommendations. The degree of labeling was determined by decomposition of the absorption spectrum using the following formula :

$$\frac{\epsilon_{SMA} \times Abs_{Atto}}{\epsilon_{Atto} \times Abs_{SMA}} \times 100$$

Measuring SMA from absorption at 260nm and Atto fluorophores at their visible absorption maximum, 500 nm and 550 nm for Atto488 and Atto532 respectively. The extinction coefficient for SMA was ϵ_{SMA} of 365$\pm$ 35 $M^{-1}_{carboxylate}$ cm^{-1}, based on absorbance of SMA dilutions, and gravimetric analysis of the dried disodium salt. The reported error includes propagated contributions from the precision of the measurements (n=3) and the documented accuracy of instruments and composition. This value is in relatively good agreement with the previously reported value of 6989 $M^{-1}_{polymer}cm^{-1}$ [28, 29], which converts using the given polymer composition to 388 $M^{-1}_{carboxylate}cm^{-1}$. The extinction coefficients of Atto dyes were ϵ_{Atto} of 9 x 10^4 $M^{-1}.cm^{-1}$ and 1.5 x

10^5 $M^{-1}.cm^{-1}$ for Atto488 and Atto532 respectively, based on the Atto-TEC literature [27].

2.3. SMALP nanodisc formation and size determination

500 μg of *E.coli* total lipid extract (Avanti Polar Lipids) were dried under nitrogen and resuspended in Tris 20 mM NaCl 200 mM with vortexing, to form multi-lamellar lipid vesicles. An appropriate proportion of SMA, or SMA-SH or fluorescent SMA, was added to the liposome solution mixed and allowed solubilize the lipids for 10 minutes. Any unsolubilized material was removed by ultracentrifugation 1 hour at $140000\times g$. The supernatant, containing nanodiscs, was collected and analyzed.

Dynamic light scattering (DLS) measurements were made on a Zetasizer Nano-series Nano-S instrument (Malvern Instruments). Samples were briefly equilibrated for 300 sec at 25°C prior to measurement. Default settings in the software were used for optimizing measurement settings and duration. Non-negative least squares algorithm of the correlation data was used to obtain volume-based particle size distributions, assuming spherically shaped particles. Volume based distributions are reported, despite the inherent assumptions, as these are easier to understand than intensity based distributions. Sample homogeneity was determined using the polydispersity index computed by the Malvern software. This index is a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis). The polydispersity index is dimensionless and scaled such that values smaller than 0.05 are rarely seen other than with highly monodisperse standards. Values greater than 0.7 indicate that the sample has a very broad size distribution [30].

For electron microscopy, carbon-coated copper grids (Electron Microscopy Sciences) were glow-discharged for 30 sec. A single droplet of 5 μL of sample was applied to the grid and removed after 60 sec by blotting with filter paper (Whatman). The grid was washed with water, and excess water was removed by blotting with filter paper prior to staining with 5 μL of 2% uranyl acetate for 60 sec. Excess of stain was removed by blotting with filter paper and the grid was dried at room temperature. Images were recorded on a FEI Tecnai 200 kV electron microscope operating at a voltage of 200 kV and a defocus of 2.5 μm, using a Eagle-CCD camera (FEI) at a nominal magnification of 50,000, yielding a pixel size of 4.4 Å.

2.4. FRET measurements

Förster resonance energy transfer (FRET) was measured using fluorescence emission spectra acquired on a Olympus confocal microscope FV1000-IX81 with a 60x oil objective controlled by Fluoview FV10-ASW4.2 software. The sample was excited at 488 nm with an Argon laser, and the emission spectrum recorded as a function of time. The data was fit by adjusting the parameters A, B and τ in the equation:

$$\begin{bmatrix} F_{525} \\ F_{550} \end{bmatrix} = \begin{bmatrix} B_{525} \\ B_{550} \end{bmatrix} + \begin{bmatrix} A_{525} \\ A_{550} \end{bmatrix} exp^{-t/\tau}$$

Where F are the fluorescence values at 2 wavelengths, B the endpoints and A the amplitudes of the fluorescence changes. This approach fits both fluorescence curves with a single rate constant.

3. Results

3.1. SMA modification

The protocol we have developed aims at obtaining thiol modified SMA (SMA-SH). For this we form an amide bond between a small proportion, typically 1%, of the maleic acid carboxyl groups and the amine groups of cystamine. Finally the di-thiol bridge is reduced using dithiothreitol (DTT) (figure 1).

In this study we have used the commercial Xiran resin with a 3:1 styrene to maleic acid ratio, however the procedure is compatible with other copolymer compositions. EDC catalyzed cross-linking is typically performed at low pH ($\approx$ 4.5) for improved efficiency but neutral pH conditions (up to 7.2) are compatible with the reaction chemistry, albeit with lower efficiency [31]. Due to the pH dependence of the solubility of SMA 3:1 the reaction was performed at pH 6.5 [32]. The solution has a tendency to change from a transparent liquid to a slightly milky gel. For reaction efficiency it was important to avoid the formation of white granular aggregates particularly during cystamine addition. To reduce precipitate formation and aid increase reaction efficiency it proved useful to increase the solvent hydrophobicity slightly, for this we added 25% ethanol.

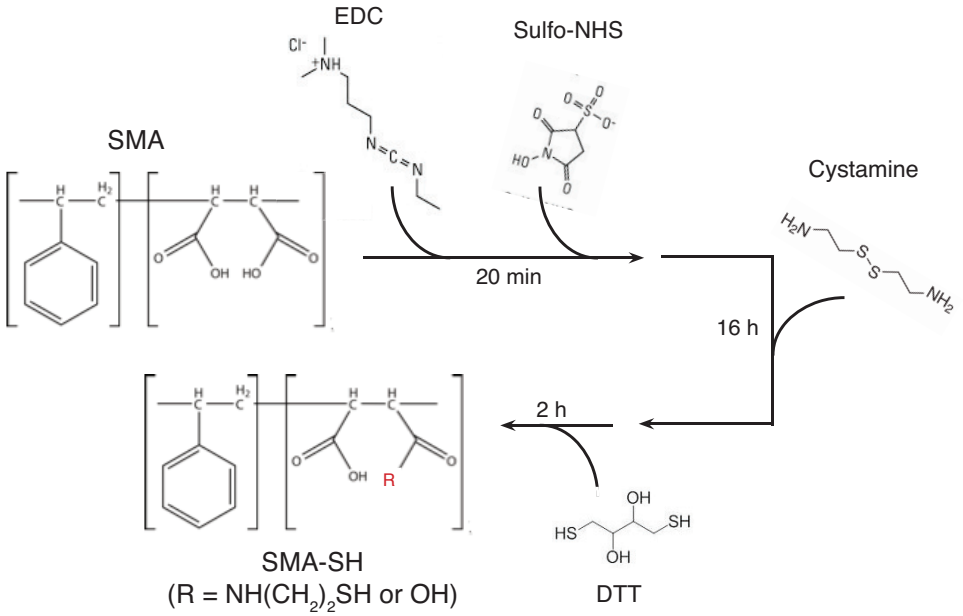

Figure 1: Reaction Scheme for the preparation of SMA-SH based on the ready-to-use Xiran SL25010 S25 from Polyscope.

3.2. Estimation of the degree of labeling

In order to estimate the degree of modification of the SMA-SH we coupled the thiol groups to fluorescent maleimides. Typically for this we used either Atto488-maleimide or Atto532-maleimide. Immediately prior to use, SMA-SH to be modified was reduced with DTT and reaction products removed by dialysis, then a 1.3 fold excess of fluorescent maleimide in dry DMSO was added. After 24 hours of reaction, unreacted fluorophores were removed using a size exclusion chromatography and labeling estimated by deconvolution of the absorption spectrum, (figure 2). For spectral deconvolution the SMA absorption maximum at 260 nm and the Atto488 absorption maximum at 500 nm, or the Atto532 absorption maximum at 550 nm, were used.

As expected, and can be readily appreciated, the modification of the SMA did not alter the UV-visible spectrum of the polymer. This is important, as we routinely estimate polymer concentration from absorption at 260 nm, using the extinction coefficient of 365 M^{-1} cm^{-1} we have determined . The Atto488 and Atto532 dyes, show little UV absorption, but have a strong visible absorption. Comparing the absorption of the maleimide and the labeled

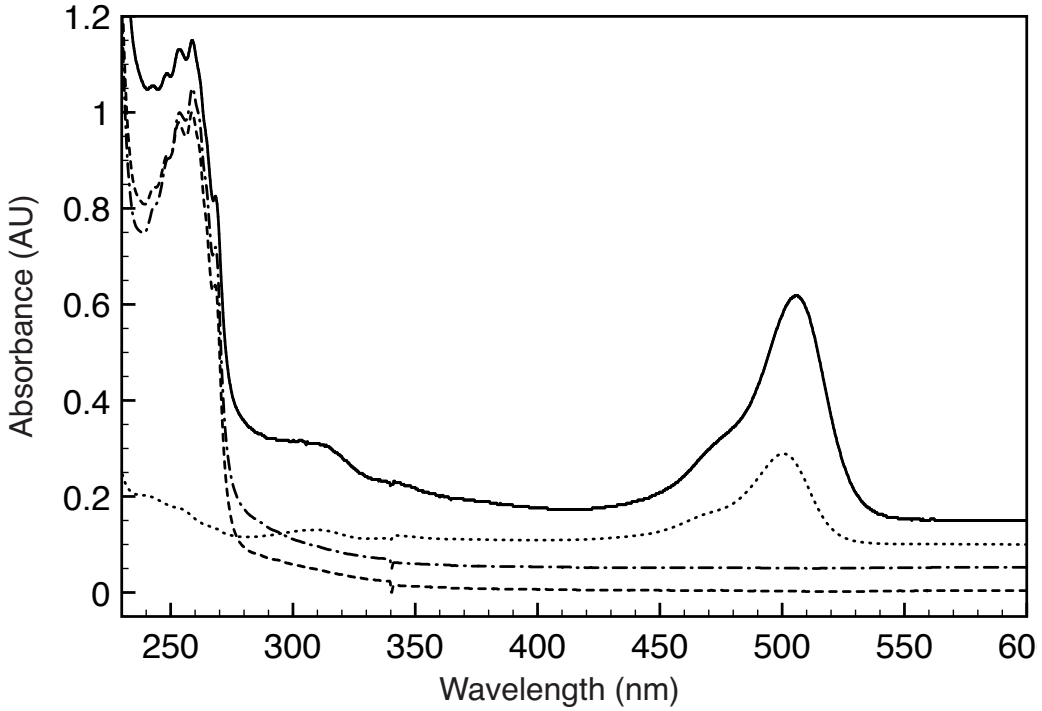

Figure 2: Absorption spectra of: SMA (dashed line); SMA-SH (dash-dotted line); Atto488 maleimide (dotted line); and Atto488 labeled SMA-SH (solid line). The various spectra are normalized and offset for improved visibility.

polymer a small red shift in the absorption maximum can be noted (from 500 to 507 nm). This small red-shift can be attributed to a change in the polarizability of the environment due to the coupling to the polymer. We have assumed for calculating degree of modification that this spectral shift is not associated with a change in the extinction coefficient.

To calculate the degree of labeling the molar ratio of dye to polymer carboxylate groups was calculated using the extinction coefficients and the fraction of maleic acid in the co-polymer, for the 3:1 copolymer we used there are 1 mole of carboxylate groups per 214.26 g of polymer. Multiple labeling reactions of the same batch of SMA-SH gave degrees of labeling between 0.16% and 0.20% for SMA-SH. At this low level of labeling the polymers have on average about 0.5 labels per chain. The final yield of labeled carboxylate groups is somewhat lower than the target, 20%, this overall yield is less than the previously reported method [22]. We believe that the EDC catalyzed derivitization is the the main reason for this low yield, longer labeling or greater excess of maleimide does not change the final yield. In particular, we suspect that this is due to working close to the solubility limit of the SMA.

3.2.1. Liposome solubilization by SMA-SH and fluorescently labelled-SMA

After performing the chemical modifications, we checked the ability of SMA-SH to solubilize liposome membranes and form SMALPs. For this multilamellar vesicles (MLV) were formed using *E.coli* total lipid extract and these were put in contact with various concentrations of polymer and the size of the resulting SMALPs was monitored using DLS (figure 3).

In agreement with previous work [33], SMALP size depends on the polymer to lipid ratio. The unmodified SMA at high polymer to lipid ratios (>3.0) gave homogeneous SMALP nanodiscs of about 7.9 nm ± 0.3 (mean ± s.d.) diameter and relatively low polydispersity 0.30 ± 0.02.

The thiol-modified polymer, SMA-SH, was slightly less efficient at solubilizing lipids, giving larger nanodiscs at all polymer lipid ratios, and with size showing a greater sensitivity to this ratio. This can be readily appreciated in figure 3A. At high polymer to lipid ratios (>3.0) nanodisc diameter was about 9.6 ± 0.3 nm. Furthermore, the SMA-SH nanodiscs were slightly more heterogeneous than those formed with unmodified polymer, as can be seen in figure 3B notably with the presence of a distinct shoulder at larger sizes. This behavior can be rationalized by the slightly more hydrophobic nature of the polymer after modification. We exclude thiol oxidative cross-linking since SMA-SH was reduced immediately prior to use.

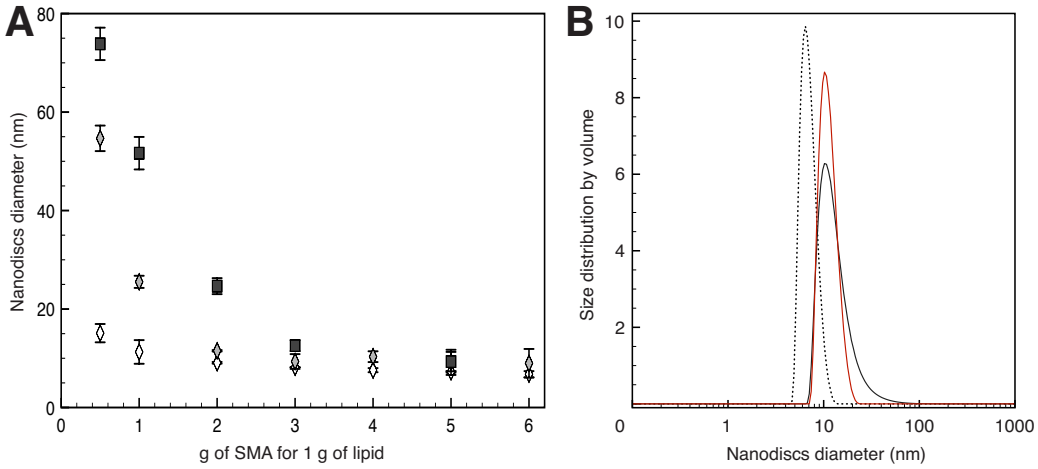

Figure 3: A. SMALP nanodisc size as a function of polymer concentration. SMALP size was determined using DLS after MLV's of *E.coli* lipids were solubilized with: SMA (open diamond); SMA-SH (gray diamond); or Atto488 labeled SMA (black square). B. DLS profiles of SMALPs formed at a polymer:lipid ratio of 3.0 *w/w* with: SMA (dashed line); SMA-SH (solid black); or Atto488 labeled SMA (red line).

Fluorescently labeled SMA was also able to efficiently solubilize MLV, figure 3A. However, it was slightly less efficient than the SMA-SH polymer. The nanodiscs formed at high polymer to lipid ratio appear to have a similar size to those formed with SMA-SH (8.0 nm $\pm$ 0.3 nm vs 9.6 $\pm$ 0.3 nm), but are slightly less heterogeneous, as can be appreciated in figure 3B. These differenes in solubilization may be due to slight modifications in the hydrophobic/hydrophilic balance.

To investigate further the heterogeneity and confirm the size difference at low polymer to lipid ratios we observed SMALPs formed at a lipid polymer ratios of 0.5 and 2.0 by transmission electron microscope (TEM), (figure 4).

The electron micrographs show clearly that at polymer:lipid ratio of 0.5 the SMA-SH objects visible are about 50 nm in diameter and much larger than the 15 nm SMA nanodiscs, visible in figure 4C and D. Gratifyingly the sizes of objects observed in electron micrographs are in agreement with those determined by DLS. The larger objects observed at low SMA to lipid ratio, particularly in the presence of SMA-SH, figure 4B, are probably a mixture collapsed polymer saturated vesicles and membrane fragments.

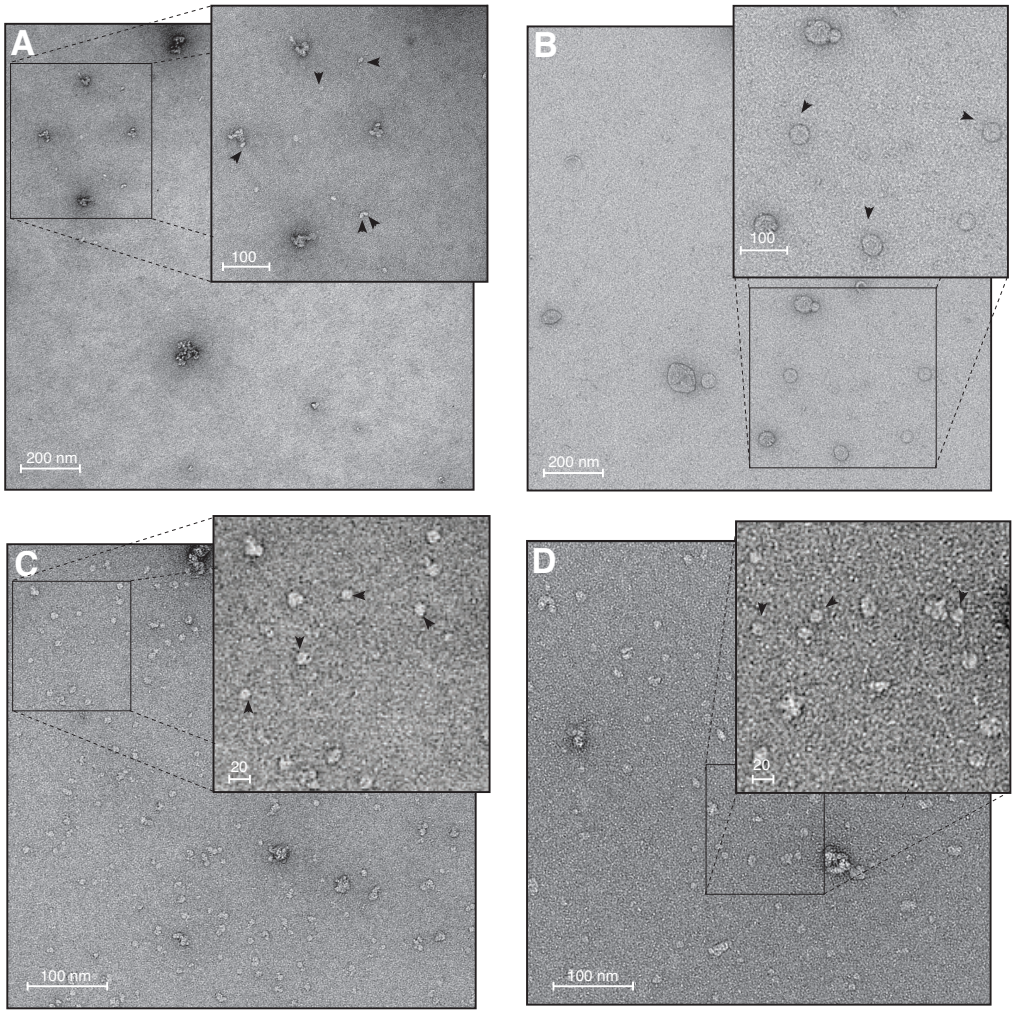

Figure 4: Negative stain transmission electron micrographs of **A)** SMA and **B)** SMA-SH nanodiscs formed at a polymer:lipid ratio of 0.5. **C)** SMA and **D)** SMA-SH nanodiscs formed at a polymer:lipid ratio of 2.0. Black arrows indicate typical individual objects in the 2 micrographs.

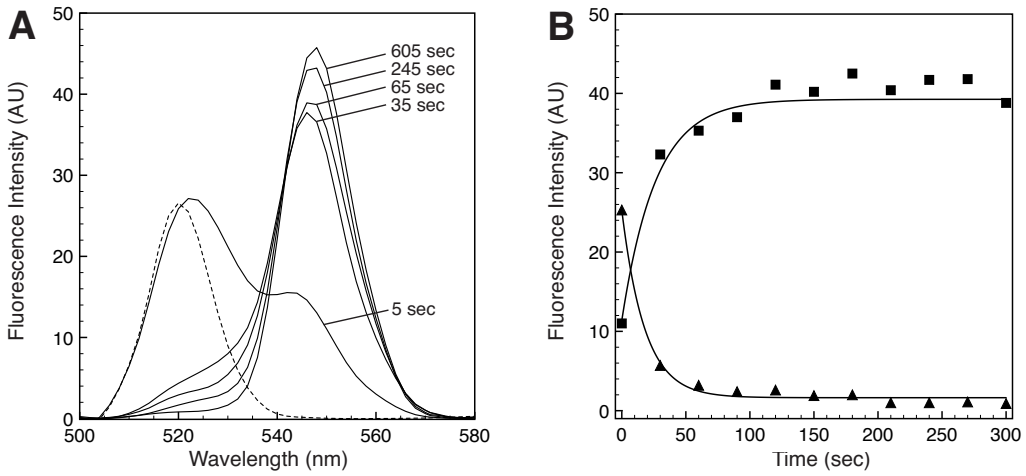

Figure 5: **A)** Emission spectra of nanodiscs labeled Atto488 (dashed black line) following mixing with and equal quantity of nanodiscs labeled with Atto532. Kinetics were recorded by measuring emission spectra every 30 seconds after sample mixing. **B)** Kinetics of FRET development followed at 525 and 550 nm. Emission at 525 nm decrease from 0 to 5 min while emission at 550nm increases. The lines show the result of a single exponential fit to the data with a time constant of 20.2 sec (see text).

3.3. Nanodisc dynamics

We have used the fluorescently labeled polymer to study the dynamics of SMALP nanodiscs. A recent study showed that lipids can exchange between nanodiscs [34]. Here we have investigated polymer exchange between nanodiscs by mixing preformed nanodiscs labeled with different fluorophores and following the changes in Förster resonance energy transfer (FRET) between the chromophores (figure 5).

In figure 5A we observe the rapid decrease in donor emission, at 520 nm, and the parallel increase in acceptor emission, at 550 nm, after mixing of the two differently colored SMALP. As the characteristic distance for transfer between these two fluorophores is 64 Å[35], and the total nanodisc concentration is low, this increase in FRET is strong evidence for exchange of polymers between nanodiscs. Development of FRET over time can be followed as seen figure 5B, and a global fit with a single exponential function to fit simultaneously the changes at both 525 nm and 550 nm gives a characteristic time of 20.2 $\pm$ 0.3 sec, this is shown as the solid lines.

It is noteworthy, that despite the low level of modification with fluorophore, about 0.5 per chain, the donor fluorescence emission declines to

near zero. This implies that each nanodisc contains multiple polymer chains, since all donors appear to be associated with acceptors after equilibration.

These measurements show that beyond the previously observed lipid exchange between SMALPs [34] there is also polymer exchange between SMALPs. These observations can explain the homogeneity of the particles produced during solubilization, since the rapid exchange of material, lipid and polymer, will ensure that the chemical potential of the different components rapidly equilibrates, resulting in homogeneous solutions.

4. Discussion

We have developed a novel protocol for the preparation of thiol labeled SMA, SMA-SH, using EDC catalysed carboxylate reaction. Furthermore, we have shown that this polymer can be derivatized to form fluorescent polymers using readily available and versatile maleimide coupling chemistry. Fluorescent SMA has been previously prepared[22], so the two protocols merit comparison. The previous protocol, based on anhydride chemistry, is cheap and has higher yields, while the procedure we report here has the advantage of using conditions compatible with most biological molecules, and reactions readily available in biology laboratories. Our method thus provides a useful alternative when low levels of modification are desired.

SMA, SMA-SH and fluorescently labeled SMA are all able to solubilize membranes with the formation of SMALP nanodiscs. The chemically modified polymers form slightly larger nanodiscs than the unmodified polymer. This is entirely consistent with the previously noted importance of the hydrophobic hydrophilic balance in modulating the energetics of membrane solubilization by SMA copolymers [32].

Since their initial appearance as solubilizing agents [36], SMA have been often been considered, or imagined, as operating like a cookie cutter, neatly excising a small disc of native membrane to form stable lipid nanodiscs [6]. Our results, and the previous results on lipid exchange [34], clearly indicate that SMALPs are much more dynamic and exchange components. Thus this cookie cutter vision is no longer tenable and a more thermodynamically driven, rather than kinetically driven, SMALP composition would seem appropriate.

Some previous clues have suggested that polymer-bound nanodiscs might be more dynamic than other system, such as nanodiscs made with membrane scaffold protein [37]. For example the potassium channel KcsA can transit

spontaneously from SMALPs to lipid bilayer [6] and a membrane protein can be transfered from SMALPs to lipid-cubic phase (LCP) in sufficient amount to obtain diffracting crystals [18].

These observations have considerable implications for the interpretation of many experiments concerning lipids and proteins in SMALPs. Previously, the vision of SMA driven membrane solubilization removing pieces of membrane, with the composition of the resulting objects being controlled by proximity in the original membrane. However, now, we must envisage a dynamic system, with the composition of the resulting objects being controlled by equilibration of chemical potential. Thus in SMALP lipidomic studies some enrichment (especially in negatively charged lipids) compared to protein-free SMALPs of solubilized *E.coli* native membranes has been observed [6][7]. Such observations can no longer be considered as a result of the native local environment but rather that the enrichment results from interactions within the SMALP population. Thus, preferential presence of certain lipids could result from favorable interactions with particular proteins, or more indirectly due to exclusion from other SMALPs in the population. The dynamic nature of SMALPs may also be useful in the pharmaceutical field as many compounds have solubility problems [38][39] we can imagine the use of SMALPs as transporters for hydrophobic drugs.

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