Protocells: Promising nanocarriers for on-demand drug delivery

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Introduction

Liposomes, lipid bilayer vesicles, are the subject of intensive research for many possible applications in drug delivery. Active pharmaceutical ingredients (APIs) are encapsulated directly in the lipid bilayer or in the aqueous solution inside of the liposome capsule. The liposome prevents a premature release of the API and also protects it from the surrounding environment. To date there are more than 10 approved formulations on the market and more than 20 other formulations are in the stage of clinical trials [1] that utilize liposomes. However, not all APIs are suitable to be formulated using liposomes due to the water solubility of the APIs, leakage through the bilayer or disruptive interaction with the lipidic membranes of the liposomes. One of the possible solutions for these problems is to first adsorb the API into a porous particle and then encapsulate this particle itself into the liposome. Such formed nanocomposites are called protocells. Protocells were firstly prepared by Ashley and Cullis [2] and later their potential was shown for on-demand drug delivery applications [3]. However, preparation of protocells is still very complicated, therefore new methods of fabrication need to be investigated before the possibility of industrial applications.

Practical part

Silica nanoparticles were prepared according to the literature [4] by the method firstly published by Stöber [5] in the 30 - 60 nm range for intravenous applications [6] and the synthesis proceeded as follows: 2.3 mL of Tetraethyl orthosilicate (TEOS) were mixed with a solution of 60 mL of ethanol, 3.0 mL of ammonium hydroxide and 1 mL of water in a 100 mL flask being heated to 60 °C. The reaction was left to proceed for 6 hours under 500 rpm stirring (stirrer length: 25 mm). The prepared dispersion was dialyzed in ethanol for 2 days during which the ethanol was changed 5 times. For better encapsulation into liposomes the nanoparticles were further aminated [7] with (3-aminopropyl)triethoxysilane (APTES) using the following procedure: 1 mL of an APTES solution (3 mL ethanol, 50 µL deionized water, 63 µL APTES, adjusted to pH 2 with hydrochloric acid, mixed for 15 minutes prior use) was added into the dispersion containing 30 mL of silica nanoparticles (Approx. 10 mg/mL) and mixed in a room temperature for 24 h. The prepared dispersion was dialyzed in water for 3 days during which the water was changed 10 times. Particles were stored in solution. The size distribution of the prepared aminated silica nanoparticles was measured by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Results of this characterization is shown in Fig. 1. Both methods show diameter of particles around 50 nm.

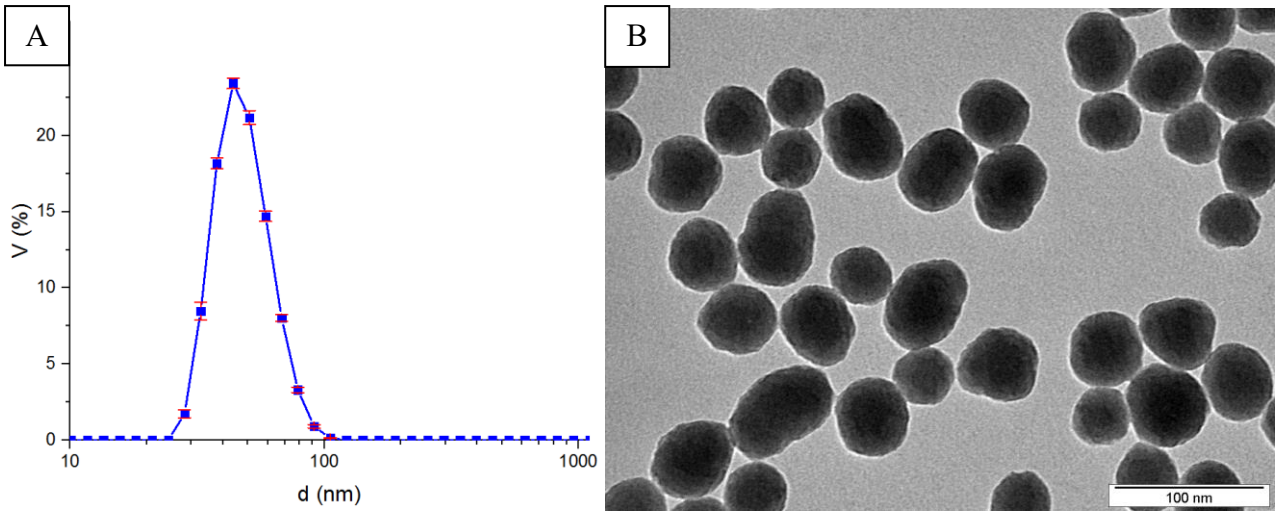

Figure 1: Characterization of aminated silica nanoparticles: A) DLS size distribution; B) TEM image

In a typical synthesis of liposomes and protocells, 10 mg of mixture of lipids and cholesterol were dissolved in 2 mL of mixture of chloroform : methanol (1 : 1) in a 25mL flask. Composition of mixture of lipids and cholesterol was chosen due to literature [8] and previous experiences. After that the solvent was evaporated in a rotary evaporator at 55 °C. The dried lipids formed a film around the bottom of the flask. The sample was then kept under vacuum for at least 6 hours. For rehydration and formation of liposomes, 2 mL of hydration dispersion containing water, NaCl, fluorescent agent 5(6)-carboxyfluorescein (5(6)-CF) and in case of protocells also silica nanoparticles dispersion was added to the flask containing the dried lipid film and the flask was vortexed until the entire lipid film was hydrated (no visible parts of the lipid film on the flask wall). Compositions of the hydration solution for each sample is shown in Table 1. The sample was then transferred to a glass syringe fitted in a liposome extrusion device heated to 69 °C and extruded through a porous membrane (pore size 800 nm) 19 times to decrease the size of the formed liposomes or protocells.

Table 1: Protocells prepared for optimization of membrane and hydrating solution composition

Sample	Liposome composition DPPC : DPPG : Chol mol %	Hydrating solution (always 2 mL)	Fluorescent agent
1	85:0:15	6 mg/mL silica	5(6)-CF 7.5 mg/mL
2	25:60:15	6 mg/mL silica	5(6)-CF 7.5 mg/mL
3	45:40:15	6 mg/mL silica	5(6)-CF 7.5 mg/mL
4	45:40:15	6 mg/mL silica, 60 mmol/L NaCl	5(6)-CF 7.5 mg/mL
5	75:10:15	6 mg/mL silica	5(6)-CF 7.5 mg/mL
6	75:10:15	6 mg/mL silica, 60 mmol/L NaCl	5(6)-CF 7.5 mg/mL

Samples 1 and 2 aggregated in the process of hydration and were not studied further. Samples 3 – 6 were successfully prepared and their encapsulation efficiency was measured by a following procedure: The sample was centrifuged (5000 RCF) and the supernatant was replaced with 60 mmol/L water solution of NaCl. This was repeated three times. Then the samples were redispersed, left for 2 hours and then again centrifuged. Fluorescent emission spectra of the supernatant were measured, then the samples were heated to 69 °C for 1 hour to promote release, centrifuged and another set of fluorescent emission spectra were measured. Amount of the released fluorescent agent was calculated from the difference of fluorescence intensity and is shown in Table 2.

Table 2: Released amounts of 5(6)-CF from samples

Sample	5(6)-CF released ($\mu g_{5(6)\text{-}CF}/mg_{lipid}$)
1	x
2	x
3	6.3
4	11.3
5	5.6
6	16.2

Results show that the composition DPPC : DPPG : Chol of 75 : 10 : 15 is slightly better than 45 : 40 : 15 and this composition of lipidic membrane was used in further experiments. Because the samples containing NaCl solution performed better, further experiments were made in the same manner. Particle size distribution and TEM images were acquired for the sample 6. (Fig. 2)

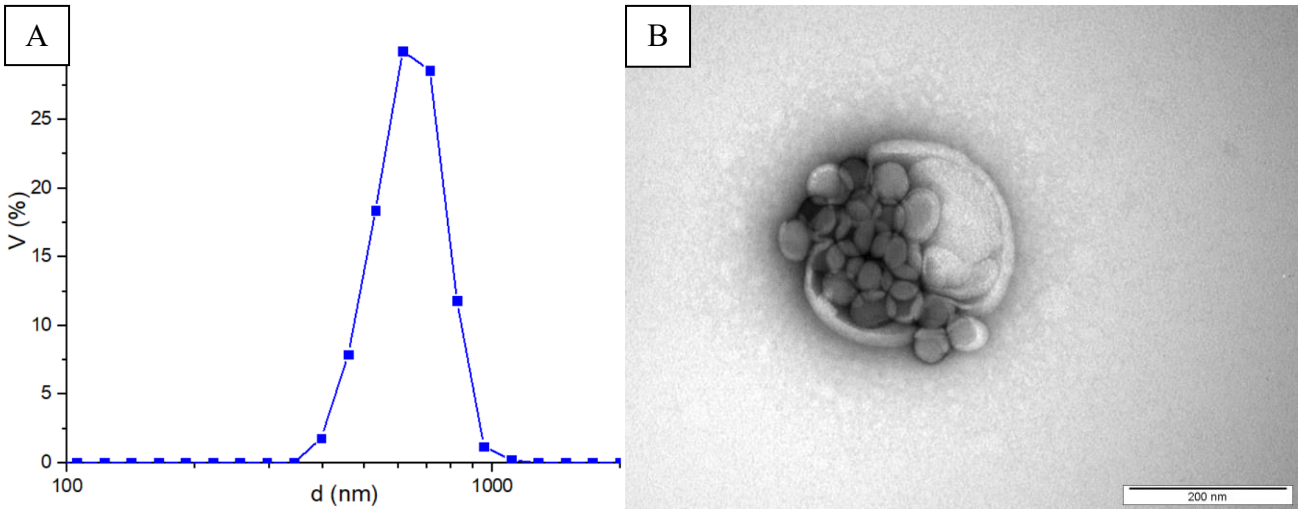

Figure 2: Characterization of protocells - sample 6: A) DLS size distribution; B) TEM image

A set of fluorescent agents was chosen for further research in the field of protocells and liposomes. To simulate various APIs fluorescein derivatives with similar structure but different behaviour (solubility, polarity) were prepared: fluorescein (F), 5(6)-carboxyfluorescein (5(6)-CF), fluorescein-5-isothiocyanate (FIC) and fluorescein-O-acrylate (FA). From all agents nearly saturated solutions in water were prepared. Mass concentrations prepared solutions are shown in Table 3.

Table 3: Solutions used as hydration solution (always with NaCl)

Fluorescent agent	Concentration / mg/mL
5(6)-carboxyfluorescein	7.5
fluorescein	2.5
fluorescein-5-isothiocyanate	0.3
fluorescein-O-acrylate	0

Fluorescein-O-acrylate proved to be so poorly soluble in water that further experiments with this agent were not conducted. Protocells and liposomes loaded with the other three fluorescent agents were prepared and the amount of the released fluorescent agents was calculated via the previously described procedure. For liposomes the maximal theoretical capacity was calculated. Results are shown in Table 4.

Table 4: Released amount from liposomes and protocells for three different fluorescent agents

Sample	Fluorescent agent (mg/mL)	Hydrating solution always 60 mmol/L NaCl in 2 mL	Released amount of fluorescent agent ($\mu g_{fluorescein}/mg_{lipid}$)	Theoretical capacity ($\mu g_{fluorescein}/mg_{lipid}$)
7	5(6)-CF 7.5	Only solution	114.5	180
8	5(6)-CF 7.5	+ 6 mg/mL of silica	16.2	x
9	F 2.5	Only solution	0	60
10	F 2.5	+ 6 mg/mL of silica	0.8	x
11	FIC 0.3	Only solution	0	7
12	FIC 0.3	+ 6 mg/mL of silica	25.2	x

The most soluble agent (5(6)-CF) (samples 7, 8) released 7 times more from pure liposomes than from protocells and the released amount is comparable to the theoretical capacity. Fluorescein (samples 9, 10) was not successfully encapsulated in liposomes and neither in protocells, although the theoretical capacity is quite high. The release of the FIC (samples 11, 12) from liposomes was below the detection limit, but was successfully released from protocells.

Conclusion

Silica nanoparticles were successfully encapsulated into liposomes with adsorbed 5(6)-carboxyfluorescein. The best composition of lipidic membrane was found for this molecule. It was also shown that encapsulation and release provide better results in environment with NaCl (aq) than in pure water. A set of protocells and liposomes loaded with three different fluorescent agents (model APIs) was prepared and their release after heating was measured. From the three molecules the Fluorescein-5-isothiocyanate provided better results when loaded into protocells compared to liposomes where no release of Fluorescein-5-isothiocyanate was observed. This suggests that protocells can be used as nanocarriers for APIs that cannot be encapsulated into pure liposomes and it may lead into reconsideration of many APIs that were in history rejected for liposomal formulation due to complications with their encapsulation.

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