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A NEW METHOD FOR RECONSTITUTION OF HIGHLY FUSOGENIC SENDAI VIRUS ENVELOPES

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A new way for reconstituting highly fusogenic Sendai virus envelopes is described. As opposed to previously described methods, in the present one the detergent (Triton X-100) is removed by direct addition of SM-2 Bio-beads to the detergent solubilized mixture of the viral phospholipids and glycoproteins, thus avoiding the long dialysis step. The vesicles obtained in the present work resemble, in their composition, size and features, envelopes of intact Sendai virus particles. The present method allows the enclosure of low and high molecular weight material within the reconstituted viral envelopes.

Introduction

Solubilization of intact animal viruses and reconstitution of their envelopes has been used as a convenient tool to study structure-function relationships of the viral envelope components [1]. Reconstituted envelopes obtained from viruses belonging to the myxovirus or paramyxovirus groups such as influenza [2] or Sendai virus [1,3,4] preserve all the functions shown by the envelope of the intact virus. Such reconstituted envelopes are able to lyse cells, to fuse with cell membranes or to induce cell-cell fusion [1–3].

Incubation of intact Sendai virus particles with non-ionic detergents such as Triton X-100 [4] or Nonidet P-40 [3] results in complete solubilization of the viral envelopes. The detergent-solubilized fractions contain both the viral phospholipids and the two envelope glycoproteins, namely the HN (hemagglutinin/neuraminidase) which mediates binding of the virus particles to cell surface receptors, and the F (fusion factor) protein whose presence is essential for promotion of virus-cell fusion [1–3]. When soluble macromolecules or membrane components are present in the detergent solubilized fractions, they are trapped within or inserted into the membranes of the vesicles formed after removal of the detergent [1,3].

Incubation of such loaded reconstituted Sendai virus envelopes with living cells at 37°C leads to fusion-mediated microinjection of the trapped material or transfer of the membrane components to the recipient cell [1,3]. Thus, besides being a useful and simple system for elucidating the detailed mechanism of virus-cell interaction and fusion, reconstituted Sendai virus envelopes offer an efficient biological carrier for transferring functional components into living cells. However, the reconstituted envelopes obtained by the existing methods suffer from the fact that they form a heterogeneous population of relatively small vesicles which are not as fusogenic as the intact Sendai virus [1,4]. Furthermore, since by existing methods the detergent is removed from the mixture of the viral glycoproteins and phospholipids by a process of dialysis [3,4], it is practically

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

impossible to trap molecules of low molecular weight within the vesicles formed after removal of the detergent. It therefore appears that an improvement of the existing methods for reconstitution of fusogenic viral envelopes would be a substantial contribution to studies of virus-cell interaction and be helpful in achieving highly fusogenic vesicles which may serve as an efficient carrier, especially for molecules of low molecular weight.

In the present work a quick and highly efficient method for obtaining a relatively homogeneous population of large Sendai virus reconstituted envelopes is described.

Materials and Methods

Removal of Triton X-100 by direct addition of SM-2 Bio-beads. Sendai virus particles were dissolved by Triton X-100, essentially as described before [4], with the following modifications: A pellet of Sendai virus particles (3 mg protein) was dissolved in 60 μl of a solution containing 10% (w/v) Triton X-100, 100 mM NaCl, 50 mM Tris-HCl (pH 7.4) and 0.1 mM PMSF (Triton/protein ratio, 2:1). After shaking for 1 h at 20°C, the detergent-insoluble material was removed by centrifugation (100000 × *g* for 60 min). The Triton X-100 was removed from the clear supernatant as follows: An amount of 40 mg of SM-2 Bio-beads was added and, after 3–4 h of incubation at 20°C with vigorous shaking, a second portion of 40 mg SM-2 Bio-beads was added concomitantly with a volume of 60 μl of Solution A (160 mM NaCl/20 mM Tricine-HCl (pH 7.4)). Following an additional incubation period of 12–14 h at 20°C with shaking, the liquid phase was then sucked by a 1 ml syringe into which the SM-2 Bio-beads cannot penetrate. The turbid suspension obtained (containing reconstituted viral envelopes) was centrifuged at 100000 × *g* for 1 h. The pellet was then suspended in 100 μl of Solution A. From each mg of intact virus particles about 300 μg of detergent solubilized glycoproteins and 80–100 μg of Sendai virus reconstituted envelopes were obtained.

Removal of Triton X-100 by dialysis in Spectraphor tubing. Sendai virus particles were dissolved in Triton X-100, after which it was removed from the detergent-solubilized fraction by dialysis in Spectraphor tubing as described earlier [1,4].

Trapping of macromolecules ([^{32}P]DNA, [^{3}H]DNA and ^{125}I-labeled IgG) within reconstituted Sendai virus envelopes. ^{125}I-labeled rabbit IgG (140 μg, $2 \cdot 10^7$ cpm/mg protein), ^{3}H-labeled calf thymus DNA (40–140 μg, 10^7 cpm/mg DNA) or ^{32}P-labeled ϕX174, RFI-DNA (70 μg, $2 \cdot 10^7$ cpm/mg DNA) were added to the detergent-soluble fraction obtained after centrifugation of detergent-insoluble viral components (2–2.5 mg of viral glycoproteins, in a final volume of 120–140 μl of 10% Triton X-100; see previous section). The subsequent steps and removal of Triton X-100 were as described above for reconstitution of unloaded Sendai virus envelopes. Externally absorbed DNA was removed from the reconstituted Sendai virus envelopes by pancreatic DNAase I (200 μg of viral glycoproteins were incubated with 200 μg of DNAase I in a final volume of 1 ml of Solution A containing 5 mM of $MgCl_2$). After 25 min of incubation at room temperature, the reconstituted Sendai virus envelopes were collected by centrifugation (100000 × *g*, 1 h). Radioactivity associated with the reconstituted envelopes was estimated by scintillation counter following their solubilization with picofluor (Packard).

Externally adsorbed IgG molecules were removed by digestion with trypsin as follows: 30 μg protein of reconstituted Sendai virus envelopes were incubated with 500 μg of trypsin (bovine pancreas, Sigma), in a final volume of 0.5 ml of Solution A, for 50 min at room temperature. Trypsinized reconstituted Sendai virus envelopes were centrifuged at 100000 × *g* for 1 h, and the pellet obtained was suspended in 40 μl of Solution A. Trapped intact IgG molecules were analyzed by 7.5% slab polyacrylamide gel electrophoresis [5].

For quantitative determination of the enclosed intact ^{125}I-labeled IgG molecules, the gels were sliced and counts in each slice (3 mm) were determined by γ-scintillation counter. Most of the radioactive material was found to be associated with the slices which were obtained from a region that should contain unhydrolyzed IgG.

Trapping of low molecular weight molecules ($Na^{125}I$ and $Na_3{}^{32}PO_4$) within reconstituted Sendai virus envelopes. $Na^{125}I$ and $Na_3{}^{32}PO_4$ were trapped within reconstituted Sendai virus envelopes as described above for IgG. Briefly, a pellet containing 6 mg of intact Sendai virus particles was dissolved

with 120 μl of 10% (w/w) Triton X-100. To the clear supernatant obtained after centrifugation, 14 μl of either 200 mM of $Na^{125}I$ (10^7 cpm/μmol) or 20 mM of $Na_3{}^{32}PO_4$ ($3 \cdot 10^7$ cpm/μmol) were added. After removal of the detergent, the SM-2 Bio-beads were washed with 240 μl of Solution A, thus giving a total volume of about 480 μl containing detergent-free reconstituted Sendai virus envelopes. Free $Na^{125}I$ or $Na_3{}^{32}PO_4$ were removed from the reconstituted envelopes by introducing a volume of 200 μl of the turbid reconstituted Sendai virus envelope suspension into 1 ml of Sephadex G-50 (Superfine, Pharmacia) columns. Fractions of 200 μl were collected at a flow rate of 200 μl/3 min by washing the column with Solution A. Protein and radioactivity of the various fractions were determined. Most of the reconstituted Sendai virus envelopes' protein was present in the third fraction, while most of the free radioactive material was in the fractions 6 and 7.

Phospholipids were determined by the method of Stewart [6] using asolectin (Associate Concentrates, U.S.A.) as a standard. Briefly, virus particles (up to 100 μg of viral phospholipids in a volume of 100 μl) were dissolved in chloroform to which ferric chloride and ammonium thiocyanate were added. After extensive vortexing, the color developed in the chloroform phase was determined at 485 nm. Protein was determined by the method of Lowry et al. [7], using bovine serum albumin as a standard.

^{125}I-labeled IgG was obtained by radioiodination of the rabbit IgG, using chloramine-T and $Na^{125}I$ as described before [8].

^3H-labeled Triton X-100 was purchased from New England Nuclear. $Na^{125}I$ and $Na_3{}^{32}PO_4$ were obtained from the Nuclear Research Center (Negev, Israel). Trypsin, PMSF, and TPCK were purchased from Sigma. Triton X-100 (average mol. wt. 500) was from Koch and Light. SM-2 Bio-beads were from Bio-Rad and used after methanol activation, as previously described [4]. Anti-Sendai virus antiserum was obtained from rabbits after injection of Sendai virus glycoproteins, and was purified as described before [9]. Rabbit IgG was purified from rabbit serum by ammonium sulfate precipitation and DEAE-cellulose column [10]. φX174, RFI-DNA was purified by the method of Clewell [11] and was nick translated with [α-^{32}P]dATP and CTP, as described before [12]. ^3H-labeled calf thymus DNA (25 μCi/ml) was obtained from New England Nuclear.

Results

Reconstitution of fusogenic Sendai virus envelopes

The main difference between the previously described methods for reconstituted Sendai virus envelopes [1,3–5] and the one described in the present work resides in the way by which the detergent (Triton X-100) is removed. In all reconstitution systems described before the detergent was removed by long dialysis [1,4], whereas in the present work it was adsorbed by SM-2 Bio-beads which were added to the detergent solubilized mixture of the viral phospholipids and glycoproteins.

Table I summarizes and compares the main features and properties of the envelopes obtained by the method described here with reconstituted Sendai virus envelopes obtained by the 'dialysis method'. As can be seen, the reconstituted Sendai virus enveloped obtained by direct addition of SM-2 Bio-beads resemble in their phospholipid/protein ratio and in their function envelopes of intact Sendai virus particles. The data in Table I confirm previously published results [1], showing that the phospholipid/protein ratio in reconstituted Sendai virus envelopes prepared by the previous method is lower than that found in the envelope of intact Sendai virus, indicating that lipid molecules are lost during the dialysis period. Those phospholipids may be essential for the viral envelope functions. The amount of Triton X-100 left in the reconstituted Sendai virus envelopes prepared by the present method is extremely low, being only 0.005% (w/v), thus giving a molar ratio of Triton to envelope phospholipid of 0.02–0.03 (Table I). Based on an average molecular weight of the viral glycoproteins of about 60 000, the calculated molar ratio of Triton to viral glycoproteins is 2–3. This is significantly lower than the amount of detergent (Triton X-100) left in reconstituted envelopes prepared by the previously described method [1].

A crowded population of reconstituted Sendai virus envelopes is seen in the electron microscope in Fig. 1A. The vesicles obtained resemble in their

TABLE I

COMPARISON BETWEEN THE PROPERTIES OF SENDAI VIRUS ENVELOPES RECONSTITUTED BY DIRECT ADDITION OF SM-2 BIO-BEADS WITH THAT RECONSTITUTED BY THE 'DIALYSIS METHOD'

Hemagglutination titer and extent of cell-cell fusion were determined by using chicken and human red blood cells, respectively, as previously described [13]. The amounts of Triton X-100 in the different preparations were determined by using [^{3}H]Triton X-100. RSVE, reconstituted Sendai virus envelope. The Triton X-100/phospholipid ratio was calculated taking the average molecular weight of Triton X-100 and the viral envelope phospholipid as 500 and 600, respectively. In addition, quantitative determination revealed that each ml of reconstituted Sendai virus envelope suspension contained 80–100 μg viral proteins and 100–120 μg phospholipids (see Materials and Methods).

System	$\frac{\text{Phospholipid}}{\text{protein}}$ (w/w)	Triton X-100 in RSVE (% w/v)	$\frac{\textit{Number of Triton X-100 molecules}}{\textit{100 molecules of phospholipids}}$	Extent of cell-cell fusion/300 HAU [a]
RSVE				
(a) Triton X-100 was removed by direct addition of SM-2 Bio-beads	1.20	0.005	2–3	+ + +
(b) Triton X-100 was removed by dialysis in Spectraphor tubing	0.40	0.020	42	+
Intact Sendai virus particles	0.35 [b]			+ + +
Envelope of intact Sendai virus [b]	1.20			

[a] HAU, hemagglutinating units.

[b] The apparently low phospholipid/protein ratio is due to the fact that intact Sendai virus particles contain six main polypeptides, while reconstituted Sendai virus envelopes contain only the two envelope glycoproteins. The figures given in the table for phospholipid/protein ratio in envelopes of intact Sendai virus particles were taken from previous work [1].

size, and especially by the appearance of highly packed populations of external spikes (arrows in Fig. 1A), the structure of intact virus particles [1,2]. Most of the vesicles are about 100–300 nm in diameter, reaching the average diameter of a population of intact Sendai virus particles [1,2] and

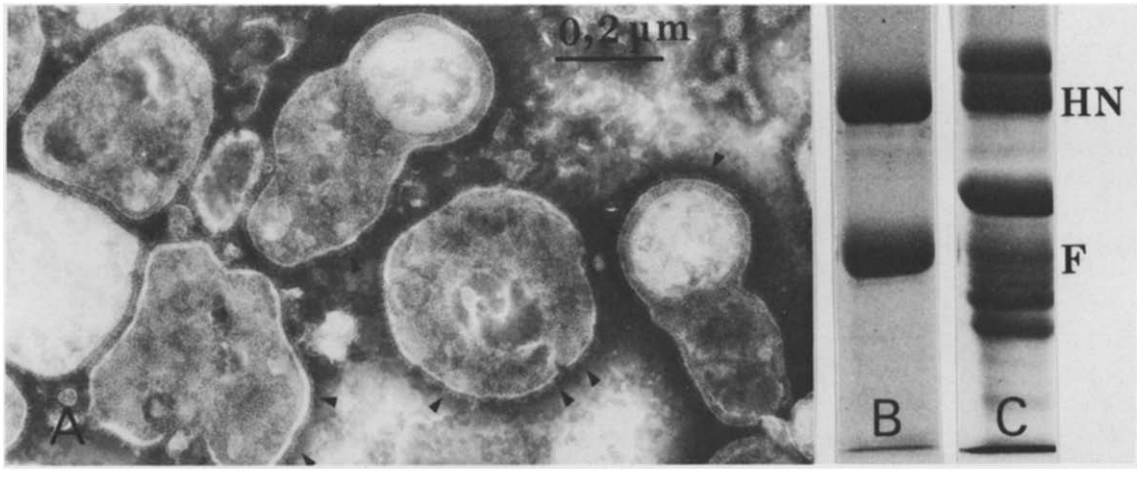

Fig. 1. Electron micrograph and gel electrophoretic pattern of reconstituted Sendai virus envelopes (RSVE). (A) Negative staining of RSVE prepared by the method described in the present work. Arrows show spikes extending from the envelopes of reconstituted Sendai virus. Electrophoretic pattern of reconstituted Sendai virus envelopes (B) or intact Sendai virus particles (C). The major Sendai virus polypeptides were classified as described elsewhere [1].

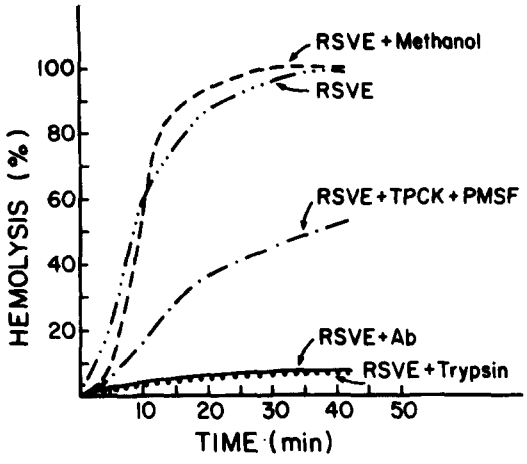

Fig. 2. Reconstituted Sendai virus envelopes induced hemolysis of human erythrocytes. Reconstituted Sendai virus envelopes (1 μg) were added to 250 μl of human erythrocytes (2.5%, v/v). Hemolysis was determined by evaluating the absorbance at 540 nm of a supernatant obtained after centrifugation of the reaction mixture, as described earlier [4]. For trypsinization, 50 μg of reconstituted Sendai virus envelopes in 100 μl of Solution A were incubated with trypsin (60 μg/ml) for 1 h at 37°C. Proteolysis was terminated by washing the reconstituted Sendai virus envelopes (100000 × g, 1 h) with cold Solution A. Treatment with PMSF (4 mM) and TPCK (10 mM) was performed essentially as described before [15], except that it was done at 37°C for 1 h. Reconstituted Sendai virus envelopes (5 μg) were incubated with anti-Sendai virus antiserum (5 μl) for 30 min at 37°C, and then 2 μg of the treated reconstituted Sendai virus envelopes were added to 250 μl of human red blood cells (2.5%, v/v).

large unilamellar liposomes [14]. Gel electrophoresis analysis showed that indeed only the two envelope glycoproteins are present in the reconstituted Sendai virus envelopes prepared by the method here (compare Fig. 1B with Fig. 1C).

Fig. 2 shows that reconstituted Sendai virus envelopes prepared by the present method are able to hemolyze human erythrocytes. Hemolysis by fusogenic viruses such as Sendai virus has been shown to reflect a process of virus-cell fusion [1,16]. This view is strengthened by the results showing that trypsinization of reconstituted Sendai virus envelopes completely abolished their hemolytic activity and pretreatment with PMSF and TPCK greatly inhibited it (Fig. 2). As expected, antiviral antibodies also inhibited the hemolysis induced by reconstituted Sendai virus envelopes (Fig. 2).

Loading of reconstituted Sendai virus envelopes with high and low molecular weight molecules

Table II shows that macromolecules such as IgG and DNA can be trapped within the reconstituted Sendai virus envelopes if added to the detergent-solubilized mixture of the viral glycoproteins and phospholipids before the addition of the SM-2 Bio-beads. About 4% of the total added IgG was trapped within the reconstituted vesicles, giving a trapping efficiency of 9.3 μg of protein/mg phospholipid or, based on the volume used, 20 μl of solute trapped/mg phospholipids. The trapping efficiency of DNA was somewhat dependent upon the molecular weight of the DNA used (not shown). When calf thymus DNA was used, about 7% of it was trapped within the reconstituted Sendai virus envelopes (4.7 μg of DNA trapped/mg phospholipid), whereas when the φX174 RFI-DNA (mol. wt. $3.5 \cdot 10^6$) was added, about 8% of it was enclosed, giving a trapping efficiency of 10 μg DNA/mg phospholipids.

Evidently, the method described in the present work allows, for the first time, to enclose molecules of low molecular weight within fusogenic reconstituted Sendai virus envelopes. The results in Table III demonstrate that when $Na_3{}^{32}PO_4$ or $Na^{125}I$ were added to the reconstitution system,

TABLE II

ENCLOSURE OF IgG AND DNA MOLECULES IN RECONSTITUTED SENDAI VIRUS ENVELOPES

Addition of ^{125}I-labelled IgG and [^{32}P]DNA to the viral envelope reconstitution system, removal of adsorbed molecules and determination of trapped radioactive material is as described in Materials and Methods.

Molecules added to the reconstitution system	μg added	μg trapped/mg phospholipids	Trapping efficiency (% of total added)
[^{125}I]IgG	140	9.3	4.0
[^{3}H]calf thymus-DNA	40	4.7	7.0
[^{32}P]RFI-DNA	70	10.2	8.0

TABLE III

TRAPPING OF SMALL MOLECULES WITHIN RECONSTITUTED SENDAI VIRUS ENVELOPES

$Na^{125}I$ and $Na_3{}^{32}PO_4$ were added to the clear solution containing the detergent solubilized viral glycoproteins and phospholipids $Na^{125}I$ and $Na_3{}^{32}PO_4$ were added to systems A and B before and after the reconstitution process, respectively. All other experimental conditions were as described in Materials and Methods.

	Molecule	μl trapped/mg phospholipids	Trapping efficiency (% of total added)
A. Added to the reconstitution system	$Na_3{}^{32}PO_4$	3.50	1.00
	$Na^{125}I$	5.00	0.70
B. Added after completion of reconstitution process	$Na_3{}^{32}PO_4$	0.40	0.02
	$Na^{125}I$	0.16	0.05

about 1% and 0.7%, respectively, of its amount was trapped within the reconstituted viral envelopes. Although the percentage of the molecules trapped seems relatively low, it appears that the trapping efficiency is reasonable, reaching up to 5 μl of solute trapped/mg phospholipids (Table III). When the $Na_3{}^{32}PO_4$ and $Na^{125}I$ were added to already reconstituted viral envelopes, namely after the detergent removal step, very little, if any, of the added molecules were found to be associated with the reconstituted Sendai virus envelopes (System B, Table III).

Table IV shows that when $Na^{125}I$ or [^{32}P]DNA loaded reconstituted Sendai virus envelopes were incubated at 4°C with human erythrocytes, about 30–40% of the radioactive material was found to be associated with the red blood cells. About the same amount of radiolabeled material was found

TABLE IV

INTERACTION OF LOADED RECONSTITUTED SENDAI VIRUS ENVELOPES WITH HUMAN ERYTHROCYTES AND EHRLICH ASCITES TUMOR CELLS

$Na^{125}I$- or [^{32}P]RFI-DNA-loaded reconstituted Sendai virus envelopes (10 μg of viral protein) or a mixture containing unloaded envelopes and $Na^{125}I$ or [^{32}P]RFI-DNA was added to human erythrocytes (2.5%, v/v) in a final volume of 1 ml of Solution A. After 10 min of incubation at 4°C and subsequent washing with Solution A (100 × *g*, 5 min), the various systems were solubilized in solubilization liquid and the radioactivity associated with the human erythrocytes was determined by scintillation counter (Packard). Association of radioactivity ([^{32}P]RFI-DNA-loaded reconstituted Sendai virus envelopes) with Ehrlich ascites tumor cells was determined essentially as described above for human erythrocytes. Briefly, [^{32}P]DNA loaded reconstituted Sendai virus envelopes (10 μg protein) or a mixture of unloaded reconstituted Sendai virus envelopes and [^{32}P]DNA were incubated with 10^7 Ehrlich ascites tumor cells (ETC) in 1 ml of Solution A at 4°C, following incubation for 20 min at 37°C. Radiolabeled material associated with the cells was determined as described above for human erythrocytes.

System	Radioactivity (% of total added) attached to		
	Human erythrocytes 4°C	ETC	
		4°C	37°C
Reconstituted Sendai virus envelopes loaded with $Na^{125}I$	30–40	n.d.	n.d.
Reconstituted Sendai virus envelopes mixed with $Na^{125}I$	1	n.d.	n.d.
Reconstituted Sendai virus envelopes loaded with [^{32}P]RFI-DNA	33	3.0	20
Reconstituted Sendai virus envelopes mixed with [^{32}P]RFI-DNA	1	2.0	2

to be associated with Ehrlich ascites tumor cells when DNA loaded reconstituted Sendai virus envelopes were incubated with these cells at 4°C. About 20% of the radiolabeled material still remained associated with the Ehrlich ascites tumor cells, even after 20 min of incubation at 37°C (Table IV). Since under these conditions the reconstituted Sendai virus envelopes fuse with the Ehrlich ascites tumor cells membranes, this radiolabeled material probably reflects DNA molecules which were microinjected into the Ehrlich ascites tumor cells cytoplasm. Experiments are currently conducted in our laboratory to demonstrate microinjection of macromolecules by more direct methods.

Discussion

An ideal biological carrier should be, presumably, a fusogenic vesicle that potentially will be able to reach a specific tissue in the living organism and subsequently fuse with its appropriate cells. In this way the content of the fusogenic vesicles, being a specific drug, antibodies or any other molecules, will be directly introduced into the cytoplasm of the recipient cells, thus making it a most effective vehicle. Although we are very far from reaching this goal, if at all possible, it seems that the use of fusogenic vesicles as carrier to transfer molecules into cells in culture is now a realistic possibility [17,18].

Loaded vesicles made up of pure phospholipids (liposomes) have already been used as drug carriers [14]. However, the fact that they are phagocytized by living cells [14] may significantly reduce their efficiency as drug or molecule carriers. Fusogenic vesicles can be prepared after solubilization of animal enveloped viruses whose infection is mediated by a virus-cell fusion step. Indeed, reconstituted viral envelopes can be prepared from viruses belonging either to the myxovirus or paramyxovirus groups and, especially, from Sendai virus particles [1,4]. This was achieved by solubilization of Sendai virus with non-ionic detergents such as Triton X-100 [4] or Nonidet P-40 [3]. However, removal of the detergent was performed in all cases by a long dialysis step. This step does not allow the trapping of molecules of low molecular weight, since the special dialysis tubings used are permeable to molecules of molecular weights below 14000 [4]. Furthermore, the long dialysis (72–96 h) required may lead to inactivation of molecules of certain biological activity such as enzymes or specific antibodies.

The present work describes a new method for reconstitution of highly fusogenic vesicles made of Sendai virus envelopes. In the method described, we have avoided the long dialysis step by addition of SM-2 Bio-beads directly to extremely small volumes of detergent-solubilized viral glycoproteins and phospholipids. Removal of detergent by this method lead to the formation of fusogenic vesicles which resemble intact virus particles in their appearance and activity much more than those described before. The method developed in the present work allows for the first time to trap molecules of low molecular weight such as $Na^{125}I$ or $Na_3{}^{32}PO_4$ within the reconstituted viral envelopes. Macromolecules such as DNA or IgG can also be enclosed within vesicles prepared by the present method, with an efficiency which is slightly higher than that obtained by the previously described techniques [19]. Preliminary experiments in our laboratory showed that such loaded vesicles can transfer biologically active material (such as inhibitors of protein synthesis, polynucleotides and polypeptides) with high efficiency to cells in culture.

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