this conventional process is referred to as "the small-scale protocol," which entails the co-incubation (loading) step and multiple steps of washing by resuspension, centrifugation and supernatant discarding, all performed in a 1-to-2-ml scale.

[0009] As follow-up to implementing the small-scale protocol, MacDiarmid *et al.* extracted drug that was associated with the minicells, see the last full sentence on page 433 *ff.*, whereupon the drug concentration was determined using HPLC analysis. For several anticancer drugs MacDiarmid *et al.* reported an estimated loading efficiency for the small-scale process in terms, for instance, of "~10 million ... molecules ... per minicell" of doxorubicin. *Id.*, first full sentence of page 435.

SUMMARY OF THE DISCLOSURE

[0010] By further investigation the present inventors have discovered that, when incubated with a nonliving, intact bacterial vesicle that intially does not contain a given fluorescent compound, the latter can move down the resultant concentration gradient (high outside to low inside) into the cytoplasm of the vesicle unexpectedly more quickly than an otherwise similar but non-fluorescent compound, and also that the fluorescent compound can achieve a surprisingly higher intra-vesicular concentration. Along these lines the inventors observed, for example, that linking a non-fluorescent compound with a fluorescent moiety greatly improves loading into nonliving, intact bacterial vesicles, relative results obtained with the non-fluorescent compound itself, notwithstanding that such modification typically effects an increase in molecular weight, which would have been thought to hamper drug loading.

[0011] In relation to the loading of fluorescent compounds particularly, the inventors also have determined that an even greater enhancement of efficiency occurs when the loading of a fluorescent compound is effected in a medium to which has been added a binary ionic compound such as an alkali metal halide salt, *e.g.*, potassium chloride, sodium chloride, and potassium bromide. Pursuant to the disclosure, the presence in the loading medium of such a compound, in a concentration as low as on the order of about 200 mM, improves loading efficiency for a fluorescent compound by two-fold or more, with loading effectively complete after only about fifteen minutes.

[0012] More generally, the inventors found that the centrifugation involved in the small-scale protocol, discussed above, can be eliminated and the small-scale protocol itelf replaced, for both fluorescent and non-fluorescent compounds, with a process of co-incubation (loading) followed by multiple steps of washing via cross-flow filtration. See, generally, CROSS FLOW FILTRATION METHOD HANDBOOK (29-0850-76 AB), GE Healthcare, *accessed at www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1392028292867/litdoc29085076_20140313045908.pdf*. See also MEMBRANE PROCESSES IN BIOTECHNOLOGY AND PHARMACEUTICS, edited by Catherine Charcosset, Elsevier (2012), and STERILE FILTRATION: A PRACTICAL APPROACH, edited by Maik W. Jornitz and Theodore H. Meltzer, Taylor & Francis (2000). In accordance with the disclosure, the cross-flow filitration is conducted with pharmaceutical grade filters, which are available commercially from Sartorius Stedim Systems, GE Healthcare and Pall Corporation, among other suppliers. It is within the purview of those knowledgeable in filtration field to select a suitable filter based on the description in the user's manual provided by the supplier, depending on the size of the filter, the production scale, etc. Additionally, the pore size of the filter is standard, *e.g.*, 0.45 μm or 0.2 μm, depending on the purpose of the filtration. The pressure applied during filtration varies at every step and is adjusted according to the need.

[0013] In this context an unexpected advantage was found to pertain when the scale of washing drug-packaged vesicles in buffer was increased, in terms of the volume of buffer employed, by between about four and five orders of magnitude, *i.e.*, on the liter scale (hereafter, "large scale") as compared to the millilitre scale of the smale-scale protocol, *e.g.*, by a three-to-five-times repeated washing of drug-loaded vesicles in about 20 liters of fresh buffer per repetition. Pursuant to this approach (hereafter, "the large-scale process"), therefore, on the order of about 100 liters of buffer can be employed, for example, in washing steps for a given batch of drug-loaded vesicles.

[0014] Consequently the inventive method yields not only a reduction in free endotoxin levels but also a reduction in a hitherto unrecognized fraction of payload compound that the conventional small-scale protocol leaves trapped on the outer surface of the vesicle. By way of illustration, the small-scale protocol resulted in a number of doxorubicin molecules loaded

per intact minicell that MacDiarmid *et al.* (2007), *supra*, estimated at about 10 million. With the large-scale process of the present disclosure, this number is under about 1 million molecules per minicell (see **Example 6** below). Thus, the small-scale protocol was associated with the trapping of some 9 million molecules of doxorubicin to the outer layer of the packaged minicells, contrasting markedly with the inventive methodology (see **Example 14**).

[0015] According to the process of the disclosure, therefore, trapping of a payload compound to the outside of a packaging vesicle is minimized, and loaded compound stays inside the vesicles when the concentration gradient is removed. The disclosure thus provides a highly effective approach to preparing intact, nonliving bacterial vesicles that enclose loaded compound in amounts on the order of hundreds of nanograms of compound per 10^9 minicells.

[0016] In one aspect, therefore, the present disclosure provides a composition comprising an intact and nonliving bacterial vesicle that encloses a fluorescent small molecule drug, which is not doxorubicin, irinotecan, bisantrene, topotecan, epirubicin, daunorubicin, mitoxantrone, Oregon Green® 488-conjugated paclitaxel, or BODIPY® FL-conjugated vinblastine. The vesicle is either an intact, bacterially derived minicell or a killed bacterial cell. In some embodiments the minicell encloses at least 500,000 molecules of the small molecule drug. Preferably, the small molecule drug is biologically active. In some embodiments the small molecule drug has a molecular weight of about 900 Dalton or less. In other embodiments, the small molecule drug is cytotoxic. Exemplary small molecule drugs include but are not limited to morpholinyl anthracycline derivatives, such as PNU-159682. In certain embodiments the small molecule drug is activiated *in vivo*.

[0017] In another aspect, the disclosure provides a composition comprising an intact and nonliving bacterial vesicle that encloses a compound of formula D-L-F or a salt thereof, wherein: D is the residue of a small molecule drug, L is a linker, and F is a fluorescent moiety. The linker suitable for this disclosure either has a half-life of between 6 hours and 24 hours or is degraded under an acidic pH condition, such as in the endosome of a mammalian cell. Illustrative small molecule drugs, fluorescent moieties, and linkers and the structures thereof are detailed below.

[0018] In yet another aspect, a composition is provided that comprises an intact, bacterially derived bacterial vesicle enclosing a compound that comprises an active agent bound through a linker to an energy transfer moiety, wherein the active agent is other than Oregon Green® 488-conjugated paclitaxel and BODIPY® FL-conjugated vinblastine. In some embodiments the energy transfer moiety is a light emitting moiety, comprises a conjugated pi system, or comprises an acridinyl moiety, a xanthenyl moiety, or a benzimidazolyl moiety.

[0019] In a related aspect, the disclosure is directed to a method of loading a plurality of minicells with a desired compound without resort to centrifugation. The method includes the steps of (A) incubating the plurality in a volume of an incubation solution of the desired compound in a buffered liquid, wherein the volume is on the order of about 100 mls or more, and then (B) subjecting the plurality to multiple washing steps, each comprising cross-flow filtration of the minicells with a volume of buffered liquid that is on the order of liters, wherein none of the washing steps employs centrifugation of the minicells. In some embodiments a binary ionic compound, which is different from the desired compound to be loaded within the minicell, is dissolved in the incubation solution to a concentration that is on the order of about 200 mM or more. Preferably, step (B) comprises three to five washing steps. In some embodiments, the desired compound is fluorescent. In some embodiments the incubating of step (A) is for a period of about 4 hours. In some embodiments the desired compound is biologically active. The desired compound can be a small molecule drug having a molecular weight of about 900 Dalton or less. Preferably, the small molecule drug is cytotoxic. The small molecule drug can be activated *in vivo*. In other embodiments the desired compound is of a formula D-L-F or a salt thereof, where D is the residue of a small molecule drug, L is a linker, and F is a fluorescent moiety. Preferably, the linker has a half-life of between 6 hours and 24 hours or is degraded in the endosome of a mammaline cell.

[0020] In yet another aspect, the disclosure relates to the use of an effective amount of a composition described herein, wherein the compound is cytotoxic, in the manufacture of a medicament for treating cancer in a patient in need thereof.

filtration of the minicells with a volume of buffered liquid that is on the order of liters, wherein none of the washing steps employs centrifugation of the minicells.

19. The method of claim 18, wherein a binary ionic compound, which is different from the desired compound, is dissolved in the incubation solution to a concentration that is on the order of about 200 mM or more.

20. The method of claim 18 or 19, wherein:

(a) step (b) comprises three to five washing steps; and/or

(b) the desired compound is fluorescent; and/or

(c) the incubating is for a period of about4 hours or more; and/or

(d) the desired compound is biologically active; and/or

(e) the desired compound is a small molecule drug of about 1500 Daltons or less; and/or

(f) the small molecule drug is cytotoxic; and/or

(g) the small molecule drug is activated *in vivo*.

21. The method of any one of claims 18-20, wherein the desired compound is of formula D-L-F or a salt thereof, wherein D is the residue of a small molecule drug, L is a linker, and F is a fluorescent moiety.

22. The method of claim 21, wherein:

(a) the linker has a half-life of between about 6 hours and about 24 hours; and/or

(b) the linker is degraded in the endosome of a mammalian cell.

23. Use of an effective amount of a composition according to any one of claims 1-17, wherein the compound is cytotoxic, in the manufacture of a medicament for treating cancer in a patient in need thereof.