MICROFLUIDIC-ENABLED SYNTHESIS OF IMMUNOLIPOSOMES

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ABSTRACT

This paper reports the first demonstration of antibody-targeted liposome (immunoliposome) formation together with in-line vesicle synthesis in a continuous-flow process. Microfluidic hydrodynamic flow focusing supports continuous-flow synthesis of unilamellar nanoscale liposomes, including the formation of tissue-targeted, ligand-bearing liposomes using pre-functionalized lipids. Though bulk-scale processes for the attachment of numerous targeting ligands (*e.g.*, antibodies, antibody fragments, proteins) to pre-functionalized liposomes have been established for tumor-specific targeting of liposomal agents, these processes require multiple vessels and long incubation periods. Here we extend the microfluidic technique for functionalized liposome synthesis to support in-line generation of nanoscale liposomes combined with post-formation conjugation of antibodies for rapid continuous-flow production of immunoliposomes.

KEYWORDS: Nanoparticles, Antibodies, Tumor-Targeted Liposomes

INTRODUCTION

Microfluidic hydrodynamic flow focusing enables continuous-flow generation of unilamellar nanoscale liposomes [1,2] and has previously been demonstrated to support the formation of tissue-targeted, ligand-bearing liposomes using pre-functionalized lipids [3]. The functionalization of nanoparticles with tissue-specific ligands is a critical step in the preparation of targeted nanomedicines, which have shown increased efficacy and reduced toxicity of the encapsulated therapeutic compounds [4].

Antibody-functionalized liposomal nanoparticles hold particular promise for enhancing therapeutic index while reducing toxicity through tissue-specific delivery, but their preparation involves an extended and multi-step process. While liposome surface functionalization may be performed during vesicle synthesis for the case of small molecule targeting ligands, post-formation functionalization is required for larger molecules such as antibodies, antibody fragments, and other proteins in order to preserve internal vesicular volume and avoid interaction of the ligands on the inner lipid bilayer leaflet with encapsulated compounds. However, post-formation ligand coupling of immunoliposomes remains a cumbersome process, requiring at least 6 different reaction vessels and long incubation periods, increasing the time and cost of the preparation process and ultimately limiting the utility of targeted liposomal drugs.

In this study, we simplify the process of post-formation liposome-antibody coupling using a microfluidic strategy. In addition to enabling rapid and automated liposome surface functionalization, the platform supports upstream synthesis of nanoscale liposomes prior to antibody conjugation for a rapid one-step and continuous-flow immunoliposome production pipeline. The developed technique represents the first demonstration of microfluidic immunoliposome formation in a single integrated and continuous-flow process. While demonstrated using cyanur attachment chemistry with whole immunoglobulin G (IgG) antibodies, the technique is amendable to other conjugation strategies and ligand types including antibody fragments and peptides, therefore enabling the production of a full range of targeted immunoliposomes within a single microfluidic platform.

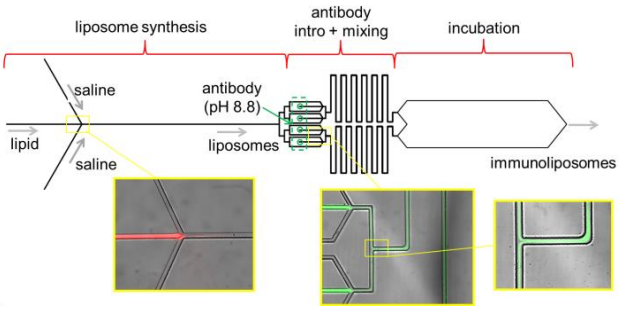

Figure 1. Schematic of the immunoliposome device, including a flow focusing region for continuous-flow liposome synthesis, a second flow focusing region with interdigitated flows for antibody introduction and pH shift, and flow expansion for on-chip incubation. Fluorescence images show flow focusing regions for both lipid and antibody introduction regions.

RESULTS AND DISCUSSION

The immunoliposome formation device reported here contains four primary stages: (1) a flow-focusing region for liposome formation, (2) ligand injection channels, (3) an interdigitated mixer to reduce diffusion lengths, and (4) a flow expansion zone to extend on-chip incubation time (Fig. 1). This design incorporates efficient liposome-antibody mixing together with a rapid pH shift between the liposome formation and coupling steps to increase reaction efficiency. To demonstrate this technique, lipids linked to cyanuric chloride [5] were used to form functionalized liposomes, followed by downstream introduction of IgG antibodies and a shift to pH 8.8 to enable rapid on-chip conjugation. The attachment process occurs via the reaction of cyanuric chloride with the N-terminus amines on antibodies or other proteins without prior derivatization [5], transpiring at a rapid rate within a microfluidic device with diminished diffusion lengths compared to conventional bulk-scale processes.

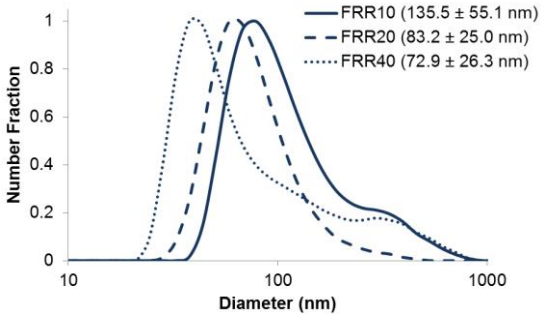

Figure 2. Size distributions for immunoliposomes generated at varying flow rate ratios (FRRs), demonstrating the ability of the microfluidic device to produce custom formations of antibody-functionalized vesicles.

To demonstrate this technique, the immunoliposome formation process was performed under various flow rate ratios (FRRs) (Fig. 2), resulting in precise size control of the liposomes while supporting the generation of liposomes under various flow conditions. The capability of the microfluidic platform to support rapid antibody conjugation was also validated through UV-vis spectroscopy (Fig. 3), revealing production of ligand-bearing liposomes with up to 2 µg/mL IgG. As the FRR increased, thus less total lipid and smaller vesicles were produced, antibody content decreased while coupling efficiency increased.

Non-specific interactions of the liposomes with the antibodies were investigated by comparing cyanur-activated and plain PEGylated liposomes. Similarly, cyanur-liposomes were compared to identical liposomes whose cyanur-antibody reactions were not quenched immediately off-chip in order to compare the difference between bulk-scale production and microfluidic generation of immunoliposomes (Fig. 4). These experiments revealed enhanced attachment of IgG to cyanur-activated liposomes compared to non-specific interactions with PEGylated liposomes lacking cyanur functional groups, and confirmed that 6 or more antibodies bound per liposome for on-chip produced immunoliposomes, comparable to immunoliposomes produced in bulk-scale processes [6]. Additionally, size distributions of each of the populations prepared under equivalent flow conditions were compared to further assess antibody conjugation efficiency (Fig. 5). Immunoliposomes produced had nearly matching modal diameters, while cyanur-free liposome produced smaller populations, suggesting an increased hydrodynamic radius due to the presence of the additional species on the immunoliposomes.

Figure 3. Antibody concentration and coupling efficiency of immunoliposomes prepared in the microfluidic process under varying FRRs.

CONCLUSION

The reported technique represents the first demonstration of microfluidic immunoliposome formation in a single integrated and continuous-flow process. The immunoliposome formation process occurs within a rapid, one-step process which is complete in seconds, generating liposomes with comparable numbers of antibodies as bulk-scale methods which take days and multiple processing steps. While demonstrated using cyanur chemistry with whole IgG molecules, the technique is amendable to other conjugation strategies and ligand types including antibody fragments and peptides. This apparatus supports various ligand conjugation strategies

and may be extended toward the formation of liposomes bearing numerous targeting moiety alternatives, including proteins and peptides which are amendable to compatible conjugation strategies. Combined with on-chip active drug loading [7], this technique could provide a means for rapid, one-step, and scalable synthesis of a wide range of fully customizable tumor-targeted therapeutic liposomes.

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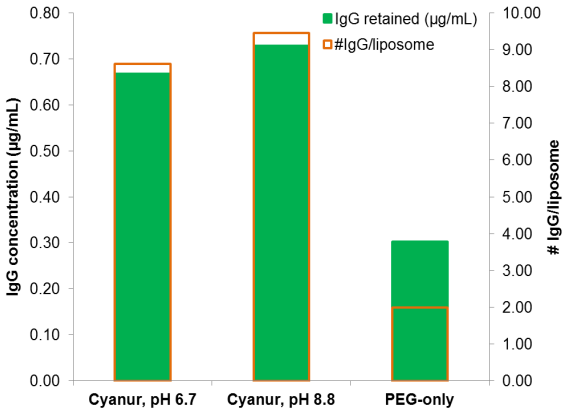

Figure 4. Antibody concentration and number of IgG molecules per liposome for immunoliposomes produced on-chip for cyanur (quenched directly off-chip, pH 6.7, and not quenched, pH 8.8) and plain PEG liposomes.

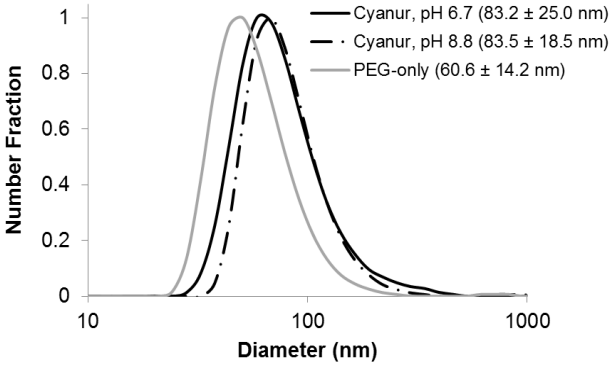

Figure 5. Size distributions of microfluidic-synthesized immunoliposomes (cyanur, pH 6.7), identical liposomes without further IgG incubation (cyanur, pH 8.8), and cyanur-free liposomes (PEG-only).

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