that relies on enzymatic polymerases to amplify low levels of nucleic acids to facilitate their detection.

Summary

[0005] The disclosure provides methods for determining, or titrating, the level of a polyanion in a sample, for example a sample comprising a Good's buffer such as MES. The sample comprises a Good's buffer (for example, a Good's buffer raw material or production lot), and may further comprise a therapeutic compound such as a biologic or a small molecule. An exemplary polyanion is polyvinyl sulfonate (*i.e.*, PVS), which is often present in varying amounts in Good's buffers or in samples of biologics at various stages of harvest and purification. These polyanions, and particularly PVS, have been found to inhibit various enzymes, including RNA and/or DNA enzymes such as polymerases. Disclosed herein are methods for detecting PVS levels in samples. The samples may comprise proteins such as biologics or may comprise other therapeutic compounds, *e.g.*, small molecule therapeutics, or both, at varying stages of production, harvest, or purification. Methods known in the art have been incapable of detecting the low levels of PVS in such samples, leading to PVS contamination of therapeutic compounds such as biologics. Such contamination can prevent authorization for use in humans and the inhibitory effects of PVS confound efforts to monitor other impurities in product preparations, such as host nucleic acids. Polyanions like PVS inhibit enzymes used in standard nucleic acid assays such as PCR, e.g., qPCR, leading to inaccurate measures of contaminating host nucleic acids. Disclosed herein is a sensitive, accurate and precise method for measuring levels of PVS, a known inhibitor of RNA enzymes, in samples comprising Good's buffer (such as protein samples) by titration. The titration methods according to the disclosure exhibit a dynamic detection range of 1.5 orders of magnitude, are highly selective for PVS relative to MES, result in a simple readout with an inflection or equivalence point providing a straightforward pass/fail output for MES buffer lots under consideration for use in monitoring host cell nucleic acid contamination of protein samples such as biologic samples. The method also lends itself to automated electrochemical or spectroscopic (*e.g.*, colorimetric, photometric, fluorometric, Raman, or FTIR spectroscopy) endpoint detection probes at reasonable cost as well as inexpensive examples reliant on standard manual titration arrangements.

[0006] In greater detail, one aspect of the disclosure is drawn to a titration method for detecting a polyanionic enzyme inhibitor in a fluid comprising: (a) contacting a fluid with a known quantity of a polycationic compound; (b) contacting the material in (a) with an indicator compound, wherein the indicator compound exhibits a changed property in the free form compared to its form when complexed to a polycationic compound, and wherein sufficient indicator compound is added to detect the free form of the indicator compound in the absence of complex formation; (c) repeating (a); and (d) detecting the free form of the indicator

compound at the titration point, thereby detecting the polyanionic enzyme inhibitor. The indicator compound may comprise or consist of an anionic indicator, including but not limited to a polyanionic indicator compound. In some examples, the fluid comprises, consists essentially of, or consists of a buffer. It is contemplated that (b) adding the indicator compound to the fluid may be performed prior to, concurrent with, or subsequent to the first iteration of (a), though it will be appreciated that in the case of subsequently added indicator compound, the indicator compound would be added before (a) is repeated. In some examples, a plurality of samples of the buffer are prepared, wherein each buffer sample has a different concentration of a buffering compound, thereby creating a dilution series of the buffer. In some examples, the limit of detection of polyvinyl sulfonate (PVS) is 1.5 parts per million of buffering solution, 0.25 parts per million of buffering solution, or 0.16 μg/mL of buffering solution. In some examples, the limit of detection of polyvinyl sulfonate (PVS) is 1.5 parts per million of buffering compound, or 0.25 parts per million of buffering compound. For example, automated methods as described herein can identify PVS at a limit of detection of 0.25 parts per million of buffering compound. In some examples, the titration end point is the point where the sample absorbance is halfway between the initial sample absorbance and the steady-state absorbance, or is a local maximum of the first derivative of the sample absorbance curve. In some examples, free indicator compound is detected electrochemically or spectroscopically. In some examples, the detection by spectroscopy comprises colorimetric detection, photometric detection, fluorometric detection, Raman, or FTIR spectroscopy. In some examples, the polyanionic enzyme inhibitor is polyvinyl sulfonate (PVS) or a derivative thereof. In some examples, the polyanionic enzyme inhibitor is polyvinyl sulfonate (PVS). In some examples, the polycationic compound is a pH-independent polycationic compound or a pH-dependent polycationic compound. In some examples, the pH-independent polycationic compound is a quaternary ammonium-based polymer. In some examples, the pH-dependent polycationic compound is a polyamine. In some examples, the quaternary ammonium-based polymer is hexadimethrine bromide (HDBr), poly(diallyl)dimethylammonium chloride (pDADMAC), or methylglycol chitosan. In some examples, the quaternary ammonium-based polymer is hexadimethrine bromide (HDBr). In some examples, a plurality of HDBr aliquots totaling at least 0.1% of a total fluid volume are added to the fluid. In some examples, the quaternary ammonium-based polymer is poly(diallyl)dimethylammonium chloride (pDADMAC).

[0007] A number of suitable indicators compounds, such as anionic indicators, may be used in examples herein. In some examples, the indicator compound is a dye, such as an azo dye. In some examples, the azo dye is Eriochrome Black T (ECBT), Eriochrome Blue Black R (Calcon) or Sulfonazo sodium salt. In some examples, the azo

dye is Eriochrome Black T (ECBT). In some examples, 0.8-1.7 µg ECBT is added per mL of the fluid comprising a known quantity of the polycationic compound. In some examples, the buffer is a Good's buffer. In some examples, the Good's buffer comprises an ethane sulfonic acid derivative or a propane sulfonic acid derivative. In some examples, the Good's buffer is MES, Bis-tris methane, ADA, Bis-tris propane, PIPES, ACES, MOPSO, Cholamine chloride, MOPS, BES, AMPB, HEPES, DIPSO, MOBS, Acetamidoglycine, TAPSO, TEA, POPSO, HEPPSO, EPS, HEPPS, Tricine, Tris, Glycinamide, Glycylglycine, HEPBS, Bicine, TAPS, AMPB, CHES, CAPSO, AMP, CAPS, or CABS. Some examples of the methods further comprise determining the concentration of the polyanionic enzyme inhibitor from the quantity of polycationic compound required to titrate the polyanionic enzyme inhibitor. Some examples of the methods further comprise comparison of the results to results obtained with a standard curve of the polyanionic enzyme inhibitor, thereby determining the concentration of the polyanionic enzyme inhibitor in the fluid. For example, the endpoint volume may be calculated for a set of multiple polyanion (*e.g.*, PVS) calibration standards (*e.g.*, 3 to 5 standards), and a standard curve may be generated. The standard curve may be used to calculate the concentration of the polyanion in a sample based on the endpoint value of the sample. Some examples of the methods further comprise performing a "limit test", in which the endpoint volume is calculated for a blank (containing no polyanion such as PVS) and a sample containing a concentration of polyanion (*e.g.*, PVS) at a specified limit. The endpoint volume of the sample may be determined, and a "pass/fail" analytical determination made based on whether or not the concentration of the polyanion in the sample is within the specified limit. In some examples, the method is automated.

[0008] Another aspect of the disclosure is directed to an automated titration method for detecting a polyanionic enzyme inhibitor in a fluid comprising: (a) combining a fluid and an indicator compound, wherein the indicator compound exhibits a changed property in the free form compared to its form when complexed to a polycationic compound, and wherein sufficient indicator compound is added to detect the free form of the indicator compound in the absence of complex formation; (b) contacting the material in (a) with a known quantity of a polycationic compound; (c) measuring the absorbance of the fluid comprising the indicator compound and polycationic compound using a titrator instrument; and (d) automatically repeating (b) and (c), wherein detection of the free form of the indicator compound detects the polyanionic enzyme inhibitor. It is contemplated that (a) combining the fluid comprising a polycationic compound and an indicator compound may be performed prior to, concurrent with, or subsequent to the first iteration of (b), though it will be appreciated that in the case of subsequently combined indicator compound, the indicator compound would be added before (b) is repeated. In some examples, the fluid comprises, consists essentially of, or

consists of a buffer. In some examples, the buffer is a Good's buffer. In some examples, the method is performed on a titrator instrument. In some examples, the titrator instrument comprises a pump such as a syringe pump or an intelligent dosing drive in fluid communication with the polycationic compound and the fluid. A suitable absorbance wavelength for the methods and systems described herein may be selected based on the indicator compound used. For example, for ECBT indicator compound, a wavelength of 660–665 nm is suitable.

[0009] Some examples include an automated titration system for detecting a polyanionic enzyme inhibitor in a fluid. The automated titration system can comprise a fluid delivery system such as a pump. The automated titration system can be configured to automatically perform a method as described herein. By way of example, the automated titration system can comprise a titrator. For example, titrators suitable for methods and systems described herein are commercially available under Metrohm's TITRANDO line of instruments. Optionally the titrator may comprise a pump for fluid communication (for example to place the polycationic compound in fluid communication with the fluid), such as a syringe pump or an intelligent dosing drive pump.

[0010] Other features and advantages of the disclosure will become apparent from the following detailed description, including the drawing. It should be understood, however, that the detailed description and the specific examples, while indicating examples, are provided for illustration only, because various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from the detailed description.

Brief Description of the Drawings

[0011] **Figure 1.** (a) Chemical structure of 2-(N-morpholino)-ethanesulfonic acid (*i.e.*, MES) shown as the acidic form, MES hydrate, and as the basic form, MES sodium salt. (b) Chemical reactions leading to compounds capable of inhibiting enzymes active on nucleic acids, such as RNA enzymes. Figure 1(b) is adapted from a figure in Smith, et al. J. Biol. Chem. 278:20934–20938 (2003).

[0012] **Figure 2.** (a) Varying the concentration of polyvinyl sulfonate (PVS) between 0-1.0 ppm revealed a linear calibration curve for two different lots of PVS standards obtained from Sigma-Aldrich, which were provided as 30 wt% aqueous solutions. The concentration of PVS was found to vary significantly lot-to-lot. It is contemplated, however, that the concentration of a particular lot can be adjusted by dilution to serve as a suitable standard. (b) Titration curves using hexadimethrine bromide (HDBr) to titrate PVS were constructed across the range of PVS concentrations of 0-1.0 ppm. A linear range of about 1.5 orders of magnitude was found.