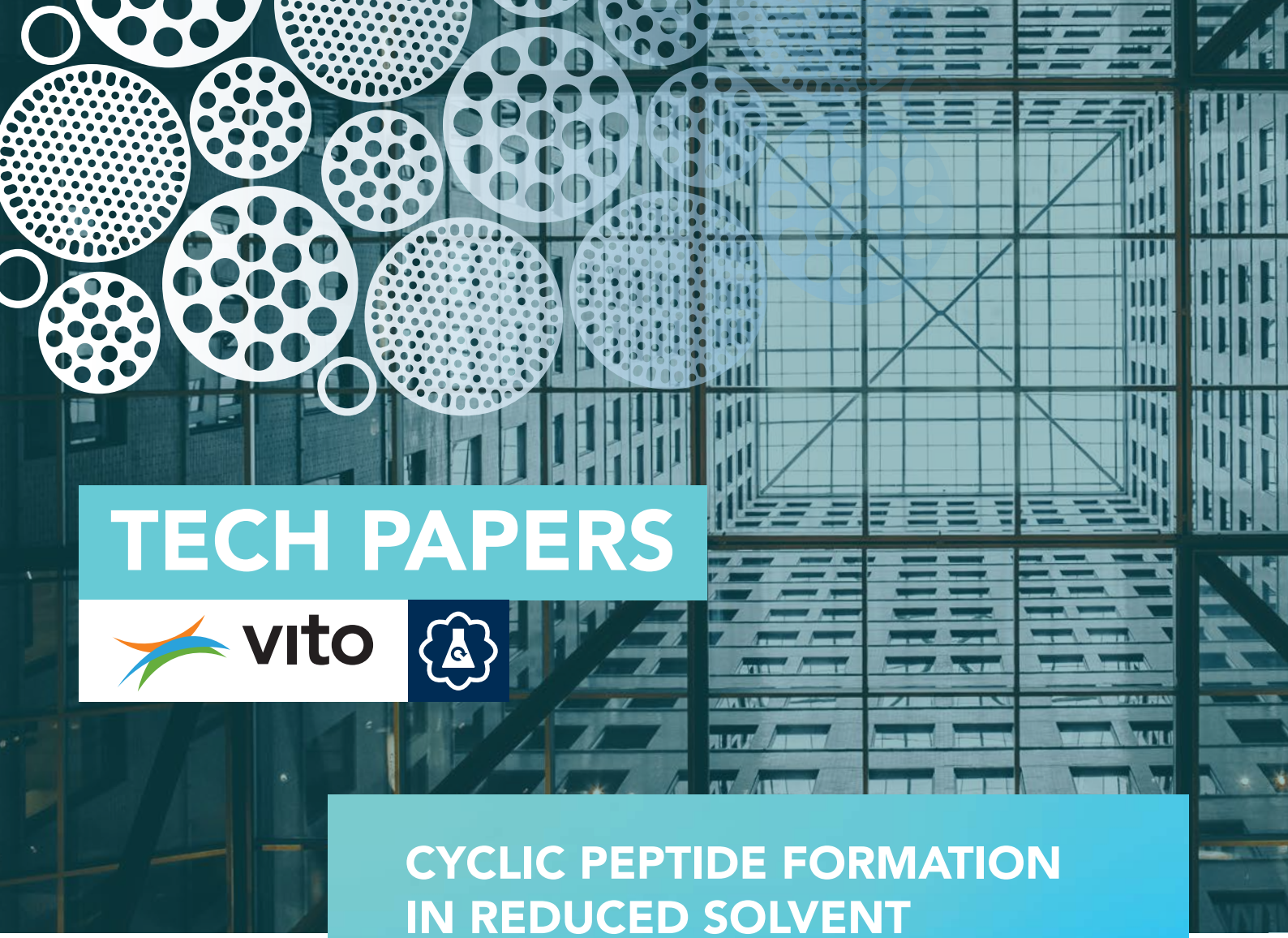

TECH PAPERS

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Dominic Ormerod, Bart Noten, Matthieu Dorec, Lars Andersson, Anita Buekenhoudt, Ludweg Goetelen

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Cyclic Peptide Formation in Reduced Solvent Volumes via In-Line Solvent Recycling by Organic Solvent Nanofiltration

Dominic Ormerod,*[,†] Bart Noten,[†] Matthieu Dorbec,[†] Lars Andersson,[‡] Anita Buekenhoudt,[†] and Ludwig Goetelen[†]

[†]VITO (Flemish Institute for Technological Research), Separation and Conversion Technology, Boeretang 200, B-2400 Mol, Belgium

[‡]PolyPeptide Group, Limhamnsvägen 108, P.O. Box 300 89, SE-200 61, Limhamn, Sweden

ABSTRACT: Cyclic peptides have found numerous and wide ranging applications that include drug molecules, nanomaterials, and chiral chromatography stationary phases. However, in the crucial cyclization step, high dilution conditions are often required, resulting in large volumes of solvent being consumed to prepare relatively small quantities of product. This paper demonstrates the synthesis of a cyclic nonapeptide with in-line solvent recycling via organic solvent nanofiltration (OSN) resulting in a significant reduction in the solvent load of the reaction and concomitant improvement in process mass intensification (PMI). The membrane was used to remove the reaction product from the reaction vessel, as the cyclic peptide product shows limited stability in the presence of an excess of reaction reagent. In comparison to the standard batch reaction, no loss in yield or product purity was observed for the OSN process tested. The proof-of-concept study outlined in this paper was performed on a real active pharmaceutical ingredient (API), and the technique used is widely applicable and flexible.

INTRODUCTION

With growing pressure being placed upon the environment and natural resources, sustainability is becoming increasingly important, within all aspects of society. This manifests itself in an increasing public awareness of the effects industry is having on the environment. The chemical industry is perceived as one of the most polluting with figures showing that the fine chemical and pharmaceutical production methods are those that produce the highest quantities of waste.[1] Reducing this waste[2] is desirable from both an economic and environmental stand point. Despite numerous successful cases of waste reduction,[3] there are still examples where improvements can be made, one such example being high dilution reactions. There are several types of reactions that must be carried out at low concentration in order to avoid formation of significant quantities of impurities. This results in large volumes of solvent being consumed in the production of relatively small quantities of the desired molecule. A typical example where such diluted conditions are required is macrocyclizations, i.e., molecules containing macrocycles that are becoming more prevalent as active pharmaceutical ingredients (APIs).[4,5] However, the large-scale production of macrocyclic molecules is made costly and problematic[6−8] by the fact that they must be performed at a low concentration in order to favor the intramolecular over the intermolecular reaction.

A potential method of confronting this problem is to apply simulated high dilution reaction conditions.[9] This technique involves the slow addition of a highly diluted solution of reaction substrate to a reactor containing a concentrated solution of reaction reagents. Though this method does permit a reduction in the solvent volumes required, compared to more conventional reactions, the solvent volumes remain high. Because solvents typically constitute 80−90% of the material input of a pharmaceutical manufacturing process, there is a clear need for a method in which these reactions can be carried out at low concentration while keeping the actual volume of solvent used to a minimum. To achieve this, a membrane assisted in-line solvent recycling methodology has been developed.[10]

Organic solvent nanofiltration (OSN) is a pressure driven filtration process capable of separating molecules in solution in the molecular weight range of 200−1000 Da.[11] This emerging technology has become more popular in the last 10−15 years since the development of membranes stable to organic solvents, some of which are now commercially available. This technique can separate solutes from solvent without the need for elevated temperatures and phase transition of the solvent from liquid to vapor phase, as occurs with more conventional methods such as distillation. Using OSN thermal degradation of high value molecules can therefore be circumvented. Furthermore, as the solvent is recycled, the need for a highly dilute solution of reaction substrate, as with the simulated high dilution conditions, is also avoidable.

Two of the parameters generally used to characterize a membrane are solute rejection and permeate flux. Rejection is a measure of the ability of a solute to permeate through the membrane. Thus, when the rejection is high, the concentration of solute in the permeate will be low. Solute rejection (R) is calculated using the eq 1 below, where C_p is the concentration of the solute in the permeate and C_r corresponds to the concentration of the solute in the retentate.

$$R = \left(1 - \frac{C_p}{C_r}\right) \times 100 \quad (1)$$

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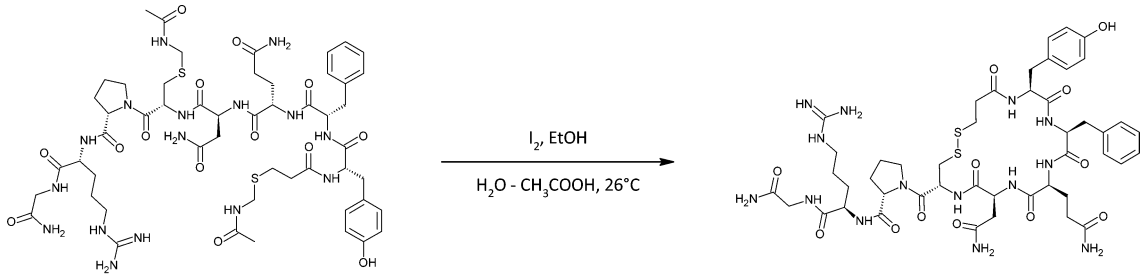
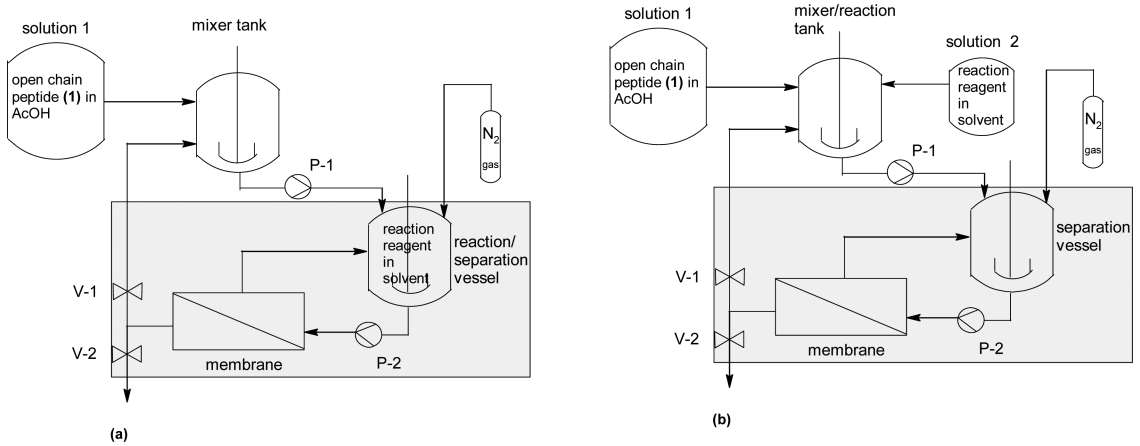
Scheme 1. Oxidative Disulfide Bridge Formation to Form the Cyclic Peptide Desmopressin



1 2

Scheme 2. Schematic Diagram of the Reactor–Membrane Configurations[a]



[a]Shaded areas are within the pressure loop and thus under pressure in use. P-1 is a diafiltration pump and P-2 a circulation pump; V-1 and V-2 are valves. Set-up (a) is the single addition mode; (b) double addition mode.

The flux (J) of solvent/solute mixtures can be determined by measuring the permeate volume (V) per unit time (t), where A is the effective membrane area, using the eq 2 below.

$$J = \frac{V}{At} \tag{2}$$

Membrane permeance (L) can further be defined as the flux as a function of trans membrane pressure eq 3.

$$L = \frac{J}{\Delta P} \tag{3}$$

The metric used to compare the batch process with this membrane assisted process was process mass intensity (PMI)[12] a mass based metric that can be calculated using eq 4: the lower the figure, the more efficient the process.

$$\text{PMI} = \frac{\text{total mass in a process or process step (kg)}}{\text{mass of product (kg)}} \tag{4}$$

The object of this work is to demonstrate that with OSN solvent recycling a significant reduction in the quantity of solvent required for a cyclization can be achieved. For this to be successful the yield and product assay must be comparable to those of the currently used batch process. The formation of a cyclic peptide has been used to illustrate the advantages of an OSN assisted technique. Additionally, among macrocycles, cyclic peptides are of particular significance due to their numerous applications ranging from drug molecules[13] to nanomaterials[14] and chiral chromatography stationary phases.[15] Several cyclic peptides are therefore produced on industrial scale often in batch processes that require extreme low concentration during the cyclization, which as a consequence can result in production bottlenecks.

The use of membranes in peptide synthesis has already been reported in the literature.[16−18] One article by Marchetti and co-workers[19] even broaches the possibility of recycling solvent. However, the publication that discusses this had no evidence this was carried out, nor would it be easy as the solvent needed purification before it could be reused. Additionally all previously published work where membranes are used in peptide synthesis refer to open chain linear peptides. In contrast, this work focuses on the successful in-line solvent recycling during the cyclization process.

RESULTS AND DISCUSSION

Reaction Studied. In order to demonstrate fully the potential of this processing method, application to an industrially relevant molecule is desirable. As such, and in collaboration with the PolyPeptide Group, the oxidative cyclization of (1−9)NH_2DDAVP **1** to the cyclic peptide desmopressin **2** was investigated. One of the reasons for choosing this molecule was that it is presently produced in a solution phase batchwise process that has been used for several years and consistently produces product of known yield and purity.[20]

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Table 1. Cyclization of 1 to 2

entry	addition mode	mol equivalents I_2	reaction temperature (°C)	yield 2 (%)	conversion 1 (%)	selectivity (%)	reduction of solvent load (%)	PMI
1	batch	1	24 ± 2	70.6	83.9	84.1	0	1703
2	single	1	24 ± 2	15.8	20.5	77.1	60	2875
3	single	1	24 ± 2	12.0	32.1	37.4	74	2216
4	single	3	24 ± 2	17.3	38.6	44.7	64	2095
5	double[a]	1	24 ± 2	42.3	49.8	85.0	63	1041
6	double[a]	1.5	24 ± 2	66.9	75.3	88.8	74	473
7	double[a,b]	1.6	24 ± 2	78.8	90.6	87.0	74	401
8	double[a]	1.5	30 ± 2	44.5	77.0	58.0	74	634
9	double[c]	1.5	24 ± 2	93	100	93	74	318
10	double[c,d]	1.5	24 ± 2	81.4	100	81.4	59	623
11	double[c,e]	1.5	24 ± 2	95	100	95	83	297

[a]Addition of iodine solution was intermittent with periods without any addition. [b]A small quantity of iodine was added to the reactor prior to commencement of reagent/substrate additions. [c]Smooth continuous addition of iodine solution. [d]Post process diafiltration of reaction product with water. [e]Filtration loop cooled to below 20 °C.

In the batch process, open chain nonapeptide **1**, which has both sulfur moieties protected by the acetamidomethyl (Acm) protecting group, is dissolved in an acidic medium and treated with 1 equiv of an ethanolic solution of iodine (I_2) (Scheme 1). The iodine both deprotects and oxidizes sulfur to form the disulfide bridge.[21] The concentration of **1** in the batch process is in the order of 1 mM, i.e., 1 g/L.

Membrane Process and Membrane Requirements. The original envisaged membrane−reactor configuration is schematically illustrated in Scheme 2a. In this set up a solution of the reaction substrate at high concentration and solvent recycled through the membrane from the reaction vessel is slowly added to a mixer tank. Through these additions a small volume of reaction substrate, at the ideal concentration for the reaction, is achieved in the mixer tank. The substrate solution is then added to the reaction vessel via constant volume diafiltration, which is the addition of a solution into the membrane filtration unit at a rate equal to the rate at which solvent is removed via permeation through the membrane (i.e., the volume within the reaction vessel is maintained constant).

For this in-line solvent recycling to be successful a membrane with high rejection for both starting material **1** and reaction product **2** is required. If this is not the case, the quantities of peptides permeating through the membrane along with the solvent will become appreciable, resulting in the loss of control over the concentration of the mixer tank solution.

For this membrane-assisted process (Scheme 2), the membrane chosen was a 50 cm, single tube, 0.9 nm TiO_2 ceramic membrane (Inopor). The initial rejection results for **1** and **2** showed this membrane to have high rejection for both molecules, 98.6% and 99.1% respectively. Thus, this remained the membrane of choice throughout the investigation.

Single and Double Addition OSN Processes. Experiments with this cyclization were initially carried out using the membrane configuration shown in Scheme 2a, where a solution of **1** in acetic acid at concentration of 19 mM (23.7 g/L) was added slowly to the mixer tank containing a mixture of water and acetic acid. The diluted solution of **1** from the mixer tank was then added to the reaction vessel which contains a solution of iodine in ethanol−water, via constant volume diafiltration. Note that the membrane flux determines the addition rate of the concentrated acetic acid solution of **1** (solution 1) into the mixer tank, with the addition rate being such that the concentration of **1** in the mixer tank never exceeded that of the batch reaction. Using the single addition mode, the reaction occurs primarily within the filtration loop, once the diluted solution of **1** comes into contact with the reaction reagent. The single addition mode was therefore never very successful. The first experiment on small scale showed that, when a 1 mol equivalent of iodine was used (similar to the batch reaction) a lower yield and conversion was achieved (Table 1, entry 2). Doubling the scale of this experiment (Table 1, entry 3), or the use of a 3-fold excess of iodine (Table 1, entry 4), resulted in higher conversion of **1** but similarly low yields of **2**. Doubling the scale of the experiment as in entry 3 results in a longer addition time for **1** and a larger excess of I_2 being present in the filtration system, particularly in the early phases of the reaction. The result of which is the formation of more secondary products. The low stability of **2** in the presence of iodine has been reported in the literature[22] and also demonstrated with the result in entry 4 of Table 1.

Further reaction of **2** to degradation products was suppressed by using a double addition mode in which both the acetic acid solution of **1** at the same concentration as previously used (19 mM) and a solution of iodine in ethanol (200 mM) were added simultaneously to the reaction tank containing a small volume of water (Scheme 2b). With the single addition mode (Scheme 2a) the reaction took place primarily within the filtration loop (shaded area in Scheme 2). Conversely in the double addition mode the site of reaction has been displaced to occur outside of the filtration loop. Again the addition rate of the solution of **1** was such that its concentration in the mixer/reaction tank never exceeded the concentration of **1** under batch conditions. The solution in the mixer/reaction tank was also added to the filtration loop via a constant volume diafiltration process.

Using the double addition mode both the conversion of **1** and yield of **2** improved compared to the single addition mode with a 1 mol equivalent of I_2, resulting in an approximately 50% conversion (Table 1, entry 5). Both yield and conversion of this reaction were however lower than the comparable batch reaction. This was because, with the membrane-assisted process, once the reaction mixture is removed from the mixer/reaction vessel very little or no further reaction appears to occur. Thus, the cyclization to **2** needs to be complete before it is added into the filtration loop, which in turn is being filled by constant volume diafiltration from the mixer/reaction vessel. Consequently the rate of which the contents are removed from this vessel is a function of the membrane flux. Therefore, the flux of the membrane determines the rate at which the reaction needs to occur. In all of the reactions reported here consistent

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permeability between 0.5 and 0.6 L m^{2-} h^{-1} bar^{-1} was observed. This was the case with two membranes of the same dimensions and pore size but from different production batches. Variation of the permeability rate can be achieved either by altering the membrane parameters (surface area, pressure etc.) or the reaction parameters. Trials were therefore carried out to increase the rate of cyclization by increasing the mol equivalents of iodine used from 1 to 1.5 (Table 1 entries 6−10). Nevertheless, achieving the target complete conversion of the starting material proved to be somewhat more complex than expected. Other methods of changing this reaction rate such as adding some iodine to the mixer/reaction tank before the addition of **1** was started (Table 1, entry 7) or warming of this tank to 30 °C (Table 1 entry 8) met with mixed success. Complete conversion was achieved using 1.5 equiv of iodine which was added to the reaction vessel concomitantly with the addition of **1**; however, the requirement being the addition must be a smooth, continuous addition. Indeed, in earlier experiments the required rate of addition of the iodine solution was sufficiently low that the addition system used tended to add this not in a smooth continuous manner but more in spurts with intermittent periods without iodine addition. Once this problem was solved complete conversion was achieved (Table 1, entry 9).

Under the conditions used in Table 1, entry 9, consistently high conversion and yield can be achieved with minimal solvent use. This cyclization has now been performed under the conditions used in entry 9 of Table 1 numerous times with consistent yield and product purity. An attempt was made to further purify the reaction product on completion of the reaction (Table 1, entry 10). Thus, a reaction was performed in exactly the same manner as previously (Table 1, entry 9). However, on completion of the reaction v-1 (Scheme 2b) was closed and v-2 opened. The contents of the filtration loop were washed with fresh water and with the intention of washing away some at least of the impurities found in the mixture. Though this appeared to have minimal effect on purity of **2**, there is some effect on yield due to some losses during the washing step, also there is obviously a direct effect on the quantity of solvent used.

Rejection and Fate of Products. As the concentration of solutes in the separation vessel increases, their rejection profile also changes to a certain extent. This resulted in a slight reduction in the rejection of both **1** and **2** (Table 2). The high rejection of the reaction starting material is the reason for the incomplete conversion when the rate of reaction is slower than the speed of diafiltration. The high peptide rejection observed can thus be used as an advantage to improve product purity. If the cyclization is complete, before reaction mixture is added to the filtration loop, via the diafiltration process, then **2** is removed from the reaction vessel, thus preventing further reaction of **2** to secondary products. The effect of preventing **2** from further reacting to secondary products can be seen in the significantly improved selectivity from experiments using the double addition mode as oppose to those from single addition (Table 1). Selectivity is simply the ratio of reaction yield to conversion expressed as a percentage. Furthermore, because of the high rejection at the end of the process practically all of **2** is to be found in the separation vessel (Scheme 2b) with the remainder being in the mixer/reaction tank.

Table 2. Rejection Profile of 1 and 2

time (h)	rejection **1** (%)	rejection **2** (%)
7	98.6	99.1
20	97.6	97.5
30	96.9	96.2

Under the double addition mode (Table 1, entry 9) the accumulation of peptide in the separation vessel over time is shown graphically in Figure 1. Evidently during the reaction time were the solutions of iodine and open chain peptide **1** are added into the mixer/reaction tank (Scheme 2b); **2** is to be found in both the filtration loop and the diafiltration tank (mixer/reaction tank, Scheme 2b). On completion of the reaction phase of the sequence, **2** in the mixer/reaction tank is transferred via the diafiltration process, which is operating under total return of the permeate, i.e., V-1 is open and V-2 closed, to the separation tank. The reaction time of 20 h is comparable to the batch process which is carried out over 16−18 h. Transfer of **2** into the separation tank was carried out by simply allowing the diafiltration process to continue; consequently, this transfer process is rather long. Obviously the duration of this process can be reduced dramatically simply by transferring all the contents of the mixer/reaction tank, once the reaction phase is complete, in one go into the separation tank.

The process conditions of Table 1, entry 9, which for this reaction gave the best result, were also carried out over an extended time period (144 h) with the intention of monitoring any changes in the peptide rejection profile. The results show the stability of both the peptide rejection and accumulation of **2** within the retentate under these conditions (Figure 1b).

A final evaluation of the rejection and flux profile of the process was performed in which the filtration loop was cooled to below 20 °C. The reasoning behind this being that reducing the temperature of the filtration step could reduce the rate of possible degradation pathways. However, reducing the temperature of the filtration would also affect the filtration parameters. The experiment was carried out as previously, i.e., a reaction phase of 20 h followed by continuation of the diafiltration to transfer **2** to the filtration loop. As can be seen graphically in Figure 2 and Table 1 entry 11, effects on yield and purity of **2** are minimal, though this reaction did give the best yield, purity of **2** and lowest PMI. Rejection also remains high as with previous reactions. The largest effect of cooling the filtration loop is on the membrane permeance which is reduced to approximately one-third of that when the filtration is carried out at ambient temperature.

Product Assay. Quantitative analysis of crude reaction product formed using the double addition OSN process shows an almost identical impurity profile as that obtained with the current batch process using the same batch of **1** as starting material. Furthermore, no impurities that were not already known from the batch process were observed when the OSN process was used (Figure 3). Indeed, a more detailed examination of the impurity profile formed during this cyclization showed a slight increase in the quantity of an impurity resulting from iodination of the tyrosine moiety of **2** but lower quantities of dimeric impurities than found in the batch process. It should also be stated that the majority of impurities found within the reaction product are impurities originating from the starting material **1** whose assay was 87%. Furthermore, post-reaction wash with pure solvent as in entry 10 of Table 1 had in this case virtually no effect on the product purity; though there remains the possibility that with a different

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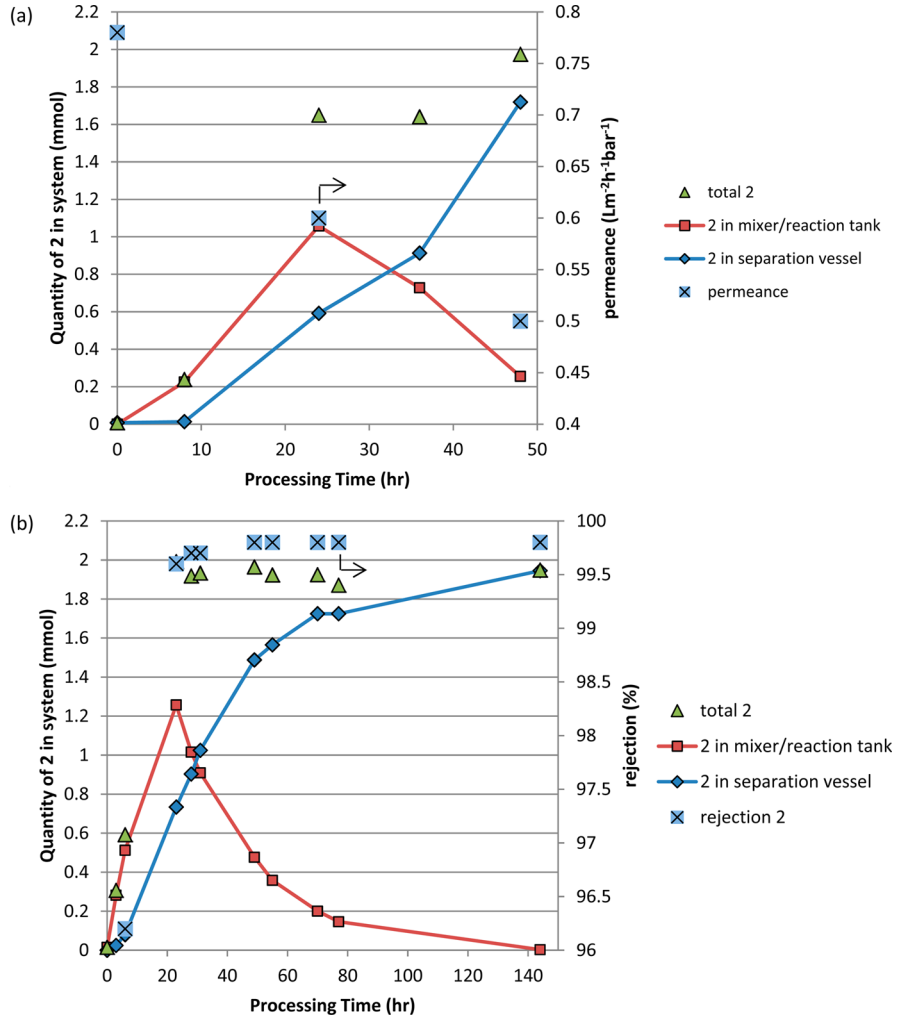

Figure 1. Fate of **2** during the process showing the accumulation of reaction product in the separation vessel. In (a) the diafiltration process was allowed to continue after the reaction phase was complete or 28 h, and in (b) this diafiltration process was performed over an extended period of time. Filtration loop in both experiments is at 24 °C.

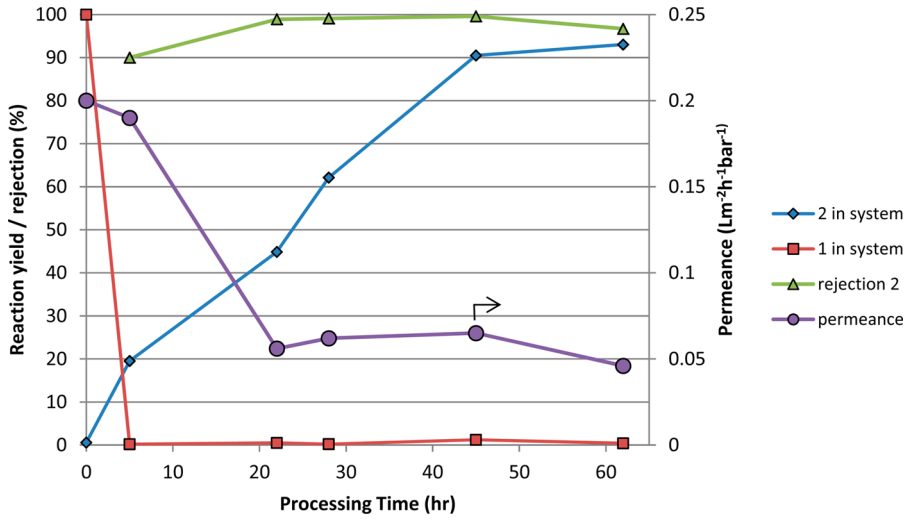

Figure 2. Rejection and flux profile for the membrane-assisted process when the filtration loop is cooled.

solvent a more positive effect on product purity could be achieved.

Reduction in Solvent Use, Comparison with a Batch Process. In order to make a meaningful comparison between this membrane-assisted processing method and the more classic batch reaction, a metric is required. The metric chosen was a mass-based metric, namely, process mass intensity (PMI). A fundamental requirement of the metric is that it is capable of

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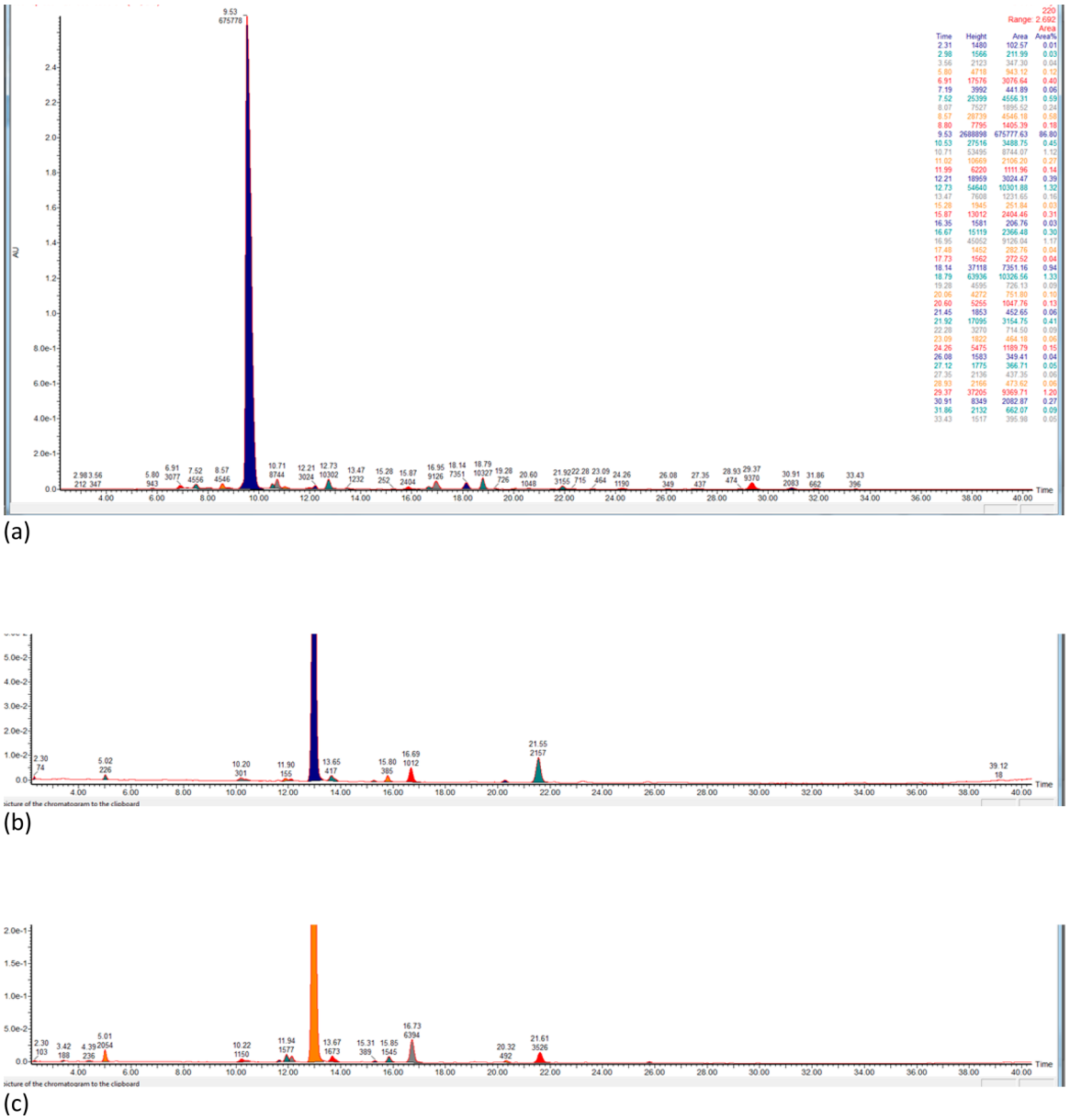

Figure 3. Chromatograms of (a) starting material **1** (b) material from a batch process and (c) material from the membrane-assisted process. Starting material **1** eluted at 9.5 min and product **2** at 12.9 min. A dimeric impurity can be seen at 21.5 min.

Table 3. Membrane Process Solvent Use

input **1** (g)	output **2** (g)	yield **2** (%)	volume solution **1** (L)	volume solution **2** (L)	volume reaction tank (L)	volume filtration loop (L)	total solvent volume (L)
2500	2200	93	117	30	188	250	585

showing the efficiency of the process. Simple reduction of the solvent volume used can be carried out in a batch process. However, this approach will lead to reduced efficiency as more byproducts will be formed. An effect completely opposite to the stated aim of this work, i.e., a decrease in solvent use while maintaining product quality, PMI is capable of demonstrating this as it takes into account the yield of the reaction. PMI can be calculated using the eq 4, the lower the figure the more efficient the process.

It is evident from Table 1 that the batch process gives a very high figure for PMI and even increases where single addition mode was used (Table 1 entries 2−4) despite a significant reduction in solvent load, thus demonstrating the low efficiency of these reactions. Significant improvements in PMI are observed once the reaction is carried out in double addition mode with PMI being enhanced by a factor of 5 for entry 9, Table 1. Even washing the reaction product with pure solvent on completion of the reaction, though this has a negative influence on the PMI is still a substantial improvement on the batch reaction.

Based on the optimal conditions from this work (Table 1, entry 9) the solvent volumes required for a double addition OSN process in which 2500 g of starting material **1** are cyclized,

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would require only 585 L of solvent (Table 3). In contrast the batch process would use 2500 L of solvent.

CONCLUSION

This work describes a new membrane-assisted processing method that enables reactions that require high dilution to be performed in significantly reduced solvent volumes, by making use of an in-line solvent recycling via OSN membranes. It has been demonstrated with cyclic peptide formation via oxidative disulfide bridge formation. Furthermore, the reaction product that in the presence of reaction reagents can further react to secondary products can be successfully produced by its continual removal from the reaction vessel. In this case it was found that the rate of reaction needs to be matched to the membrane permeance, a variable that can be controlled. Solvent load in the reactions can be reduced by up to 83% with no detrimental effects on reaction yield or product purity. Also, this processing method is capable of producing 5 times as much product as is presently produced in the existing large-scale reactors or if production quantities are to be maintained at the present levels it can be produced in reactors of smaller footprint. Additionally, no new reactors or adaption of existing reactors is required; a nanofiltration unit is a stand-alone unit that can be connected to existing reactors via standard connections. This proof-of-concept study achieved its aims in a relatively short period of time, measured in weeks. Though scale-up as is of the process developed in this proof of concept study is possible, further improvements can still be made to achieve an even more performant process.

EXPERIMENTAL SECTION

Materials. The solvents used in this study were ethanol, acetic acid, and water. Ethanol and acetic acid were technical grade purchased from VWR (Belgium) and used without prior purification. Water was reverse osmosis purified water. Iodine (ACS reagent) was purchased from Sigma-Aldrich (Belgium) with an assay of 99.8% and used with no further purification. Cyclisation precursor (1−9)NH_2DDAVP **1** and analytical standard of **2** were donated by PolyPeptide Group (Malmö, Sweden). The membrane used in this work was a asymmetric tubular 0.9 nm TiO_2 membranes; length 50 cm, outer diameter 1 cm, inner diameter 0.7 cm, and top layer thickness of about 50 nm, purchased from Inopor (Veilsdorf, Germany). All membrane experiments were performed in a cross-flow nanofiltration unit made in house, pressurized with nitrogen gas. The filtration was performed with a cross-flow velocity of 2 m/s and a transmembrane pressure of 10 bar.

Analysis. Quantitative analysis of samples was performed in two ways (a short method and long method). The short method of 11 min used an UPLC with a Waters Acquity UPLC system equipped with a photodiode array (PDA) detector. A Waters Acquity BEH C18 column of dimensions 2.1 mm × 100 mm, 1.7 μm was used with a column temperature of 40 °C. A portion of 10 μL of sample was injected on the column with a mobile phase of 10 mM ammonium acetate in water and acetonitrile in a gradient. The detector was used at wavelengths of 204 and 276 nm. This shorter method was used to follow the progress of the reaction during the process. For a more in-depth impurity profile of the crude reaction product or mixtures a longer method of 40 min was used, also on the Waters Acquity UPLC system equipped with a photodiode array (PDA) detector. An Agilent Zorbax Eclipse XDB-C18 column of dimensions 4.6 mm × 150 mm, 5 μm was used with a column temperature of 40 °C. 10 μL of sample was injected on the column with a mobile phase of an aqueous phosphate buffer and acetonitrile in a gradient. The phosphate buffer was prepared from 3.52 g of KH_2PO_4 and 7.3 g of $Na_2HPO_4{\cdot}2H_2O$ in 1 L of water. The detector was used at wavelength of 220 nm.

Cyclisation Reactions. *a. Batchwise.* Under an atmosphere of nitrogen, acetic acid (125 mL) was added to a reaction vessel containing water (1000 mL) and the temperature adjusted to 26 °C. (1−9)NH_2DDAVP **1** (1.25 g, 1 mmol) was added and the mixture stirred at 26 °C until complete dissolution. A solution of iodine (254 mg, 1 mmol) in ethanol (10 mL) was added dropwise to the peptide solution over 6 h. The resulting mixture was stirred at 26 °C for a further 12 h. Progress of the reaction was followed by UPLC analysis.

b. Membrane-Assisted, Single Addition Mode. 400 mL of water and a solution of iodine (510 mg, 2 mmol) in ethanol (20 mL) was added to the filtration loop fitted with a membrane. The resulting mixture was circulated through the system at atmospheric pressure until the internal temperature was 26 °C. Connected to the filtration unit via a pump and set up to perform constant volume diafiltration was a 300 mL of stirred solution of acetic acid (33 mL) in water (267 mL); this is the diafiltration solution. The filtration loop was brought under 10 bar pressure with nitrogen and the membrane flux continually monitored. Permeate from the membrane was added directly to the diafiltration solution. A solution of (1−9)NH_2DDAVP **1** (2.5 g, 2 mmol) in acetic acid (111 mL) was added to the diafiltration over 12 h (using a syringe pump). On completion of the addition of the peptide solution the diafiltration was allowed to continue a further 16 h with regular sampling of the filtration loop contents (retentate), the diafiltration solution, and the membrane permeate outlet (permeate) for analysis.

c. Membrane-Assisted, Double Addition Mode. 400 mL of water was added to the filtration loop, fitted with a membrane, and circulated through the system at atmospheric pressure until the internal temperature was 26 °C. Connected to the filtration unit via a pump and set up to perform constant volume diafiltration was a 300 mL stirred solution of acetic acid (33 mL) in water (267 mL); this is the diafiltration solution. The filtration loop was brought under 10 bar pressure and the membrane flux continually monitored. Permeate from the membrane was added directly to the diafiltration solution. To the diafiltration solution was added via a syringe pump over 18.5 h a solution of (1−9)NH_2DDAVP **1** (4.0 g, 3.2 mmol) in acetic acid (177.8 mL). From a second syringe pump was added a solution of I_2 (1.21 g, 4.8 mmol) in ethanol (47.7 mL) over 19 h. On completion of the addition of the peptide solution the diafiltration was allowed to continue a further 16 h with regular sampling of the filtration loop contents (retentate), the diafiltration solution, and the membrane permeate outlet (permeate) for analysis.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +32 14 33 5650. Fax: +32 14 32 1186. E-mail: dominic.ormerod@vito.be.

Notes

The authors declare no competing financial interest.

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