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Insights into synergistic interactions in binary mixtures of chemical permeation enhancers for transdermal drug delivery

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Abstract

Chemical permeation enhancers (CPEs) are known to increase skin permeability to therapeutic drugs. Single chemicals, however, offer limited enhancements of skin permeability. Mixtures of chemicals can overcome this limitation owing to their synergistic interactions. However, identification of potent mixtures of chemicals requires screening of a large number of formulations. Discovery of CPE mixtures can be significantly accelerated by identifying patterns that occur in the existing data on CPEs. In this study, we systematically mine through a huge database on skin permeabilizing effect of over 4000 binary formulations generated by high throughput screening and extract general principles that govern the effect of binary combinations of chemicals on skin's barrier properties. Potencies and synergies of these formulations are analyzed to identify the role played by the formulation composition and chemistry. The analysis reveals several intuitive but some largely non-intuitive trends. For example, formulations made from enhancer mixtures are most potent when participating moieties are present in nearly equal fractions. Methyl pyrrolidone, a small molecule, is particularly effective in forming potent and synergistic enhancer formulations, and zwitterionic surfactants are more likely to feature in potent enhancers. Simple but invaluable rules like these will provide guiding principles for designing libraries to further speed up the formulation discovery process.

Keywords: Synergy; Skin; Library Design; Combinations; High throughput screening

1. Introduction

Low permeability of skin to drugs poses a significant bottleneck in the development of transdermal and topical therapies [1]. Evolved to impede the flux of toxins and xenobiotics into the body, skin naturally offers a very low permeability to the movement of foreign molecules across it [2]. Several approaches including the use of physical techniques such as iontophoresis [3], sonophoresis [4], microneedles [5], electroporation [6] and photoacoustic waves [7] as well as chemical techniques based on permeation enhancers [8] have been adopted to enhance skin permeability to drugs.

Chemical enhancers have a long history of use in transdermal and topical drug delivery applications [9,10]. Some of the notable examples of chemical enhancers include fatty acids (for example, oleic acid [11,12]), fatty esters (for example, isopropyl myristate [13,14]) and solvents (for example, dimethyl sulfoxide). A large number of studies have shown that CPEs enhance skin permeability to drugs, especially small and lipophilic candidates [15–21]. The overall effectiveness of CPEs, however, has been moderate, especially in comparison to physical approaches. Another issue with CPEs has been the safety concerns associated with their use, for example skin irritation [22], thereby limiting their use. Pushing the envelope on enhancement effectiveness with single enhancers often leads to a compromise on safety issues. Nevertheless, interest in CPEs has continued to build due to their low cost, ease of use and simplicity.

Limitations of individual CPEs can be potentially overcome by using mixtures of two or more CPEs. Towards that end, several studies have reported on combinations of one or more enhancers for drug delivery [18,23–37]. Although CPE mixtures provide an attractive alternative compared to individual chemicals, this advantage is almost immediately offset by the enormity of the parameter space. For example, random selection of two CPEs from a list of ~300 chemicals leads to over 40,000 binary pairs and if each pair was explored even at a modest 25 different compositions, the number of formulations in the library approaches a million. Testing such a huge library for its effect on skin permeability is hindered by the slow test speeds of Franz diffusion cells which are commonly used for formulation screening.

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High throughput screening methods have been recently introduced to facilitate screening of transdermal formulations [38]. Using this method, we have screened several thousand binary formulations and reported on a few peculiar ones that led to significant enhancement of skin permeability [38]. Although high throughput screening methods have brought about a 100-fold improvement in screening rates, it is still challenging to search through the entire formulation space of binary formulations. The limitations of throughput become more critical for formulations containing more than two CPEs. It is clear that high throughput screening methodologies can benefit by strategies to streamline the parameter space. With this in mind, we mined the database generated by screening over 4000 binary formulations prepared by systematically combining 32 distinct CPEs in a common solvent. Particular emphasis was placed on studying synergistic combinations of CPEs, that is, formulations whose activity (enhancement of skin permeability) is higher than the average of activities of the individual ingredients. Importance of synergistic activities owes to two reasons. First, as will be shown later, synergistic formulations usually lead to higher transport enhancements. Second, synergistic effects of CPEs are difficult to predict from first principles and experimental exploration is the only method of identifying them. Accordingly, guidance from existing experimental data will be helpful in identifying future synergistic combinations. We specifically sought to answer questions such as how commonly do synergies occur among CPEs, are certain compositions more likely to yield high synergies and, more importantly, are certain CPEs more likely to yield high synergies?

2. Methods and materials

2.1. Library generation

Formulation library consisted of 32 CPEs which were classified into eight categories: (i) *anionic surfactants* (SLS, NLS, SOS, SLA), (ii) *cationic surfactants* (CTAB, DPC, BDAC, OTAB), (iii) *zwitterionic surfactants* (HPS, CBOL, CBC, CBCAS), (iv) *nonionic surfactants* (T20, S20, PEGE, TR), (v) *fatty acids* (OA, LIN, LOA, LA), (vi) *fatty esters* (TET, IM, SO, ML), (vii) *azone-like compounds* (NDP, DA, PP, NS) and (viii) *others* (MEN, MP, CIN, LIM). The category of azone like compounds is defined to include amines and molecules containing N and O in a ring structure. For full names of the chemicals and their CAS numbers, refer to Table 1. SLS, NLS, SOS, CTAB, DPC, BDAC, OTAB, HPS, T20, S20, TR, OA, LIN, LOA, LA, TET, IM, ML, NDP, DA, NS,

Table 1
List of enhancers used in this study

No.	Group	Name (common name)	Abbr.	CAS No.	Category
1	I	Sodium lauryl sulfate	SLS	151-21-3	AS
2		Cetyl trimethyl ammonium bromide	CTAB	57-09-0	CS
3		Dimethylpalmityl ammoniopropane sulfonate	HPS	2281-11-0	ZS
4		Oleic acid	OA	112-80-1	FA
5		Tetracaine hydrochloride	TET	136-47-0	FE
6		Decyl pyrrolidone	NDP	55257-88-0	AZ
7		Menthol	MEN	89-78-1	OT
8		Poly(oxyethylene) sorbitan monolaurate	T20	9005-64-5	NS
9	II	*N*-Lauroyl sarcosine sodium salt	NLS	137-16-6	AS
10		Dodecyl pyridinium chloride	DPC	104-74-5	CS
11		Oleyl betaine	CBOL	871-37-4	ZS
12		Linoleic acid	LIN	60-33-3	FA
13		Isopropyl myristate	IM	110-27-0	FE
14		Dodecyl amine	DA	124-22-1	AZ
15		Methyl pyrrolidone	MP	872-50-4	OT
16		Sorbitan monolaurate (Span 20)	S20	1338-39-2	NS
17	III	Sodium octyl sulfate	SOS	142-31-4	AS
18		Benzyl dimethydodecyl ammonium chloride	BDAC	139-07-1	CS
19		Cocamidopropyl hydroxysultaine	CBCAS	068139-30-0	ZS
20		Lauric acid	LA	143-07-7	FA
21		Sodium oleate	SO	143-19-1	FE
22		Nicotine sulfate	NS	65-30-5	AZ
23		Cineole	CIN	470-67-7	OT
24		Polyethylene glycol dodecylether (Brij® 30)	PEGE	9002-92-0	NS
25	IV	Sodium lauryl ether sulfate (SulfoChem ES-1®, $n=1$)	SLA	68585-34-2	AS
26		Octyl trimethyl ammonium bromide	OTAB	2083-68-3	CS
27		Cocamidopropyl betaine	CBC	61789-40-0	ZS
28		Linolenic acid	LOA	463-40-1	FA
29		Methyl laurate	ML	111-82-0	FE
30		Phenyl piperazine	PP	92-54-6	AZ
31		Limonene	LIM	5989-27-5	OT
32		Triton®-X100	TR	9002-93-1	NS

Thirty-two CPEs were selected from a pool of over 200 molecules that represent chemicals used in patches or topical formulations at clinical or experimental level. Four representatives were picked from each of the eight categories including anionic surfactants (AS), cationic surfactants (CS), zwitterionic surfactants (ZS), nonionic surfactants (NS), fatty acids (FA), fatty esters (FE), azone-like compounds (AZ) and others (OT).

MEN, MP, CIN and LIM were purchased from Sigma Chemicals (St. Louis, MO). SO, PP and PEGE were obtained from TCI America (Portland, OR). CBOL, CBC, CBCAS and SLA were obtained as gift samples from Chemron Corp. (Paso Robles, CA). To limit the size of the library, CPEs were divided into four blocks such that each group has one representation from each class of enhancers (see Table 1). Enhancers within each block were paired to generate 28 binary combinations (a total of 112 combinations over the entire set under study). For each pair of enhancers, four different total concentrations of 0.5%, 1%, 1.5% and 2% w/v were selected.

At each total concentration, the weight fraction of one enhancer was varied from 0 to 1 in steps of 0.1. Thus, for each enhancer pair, 44 test formulations were generated, 36 of which contained two components. Thus, a total of 4032 (112×36) binary formulations were studied. All formulations were prepared in a 1:1 phosphate buffered saline/ethanol (PBS/EtOH) solution. This solvent system was chosen for its compatibility in solubilizing all enhancers used in this study. 1:1 PBS/EtOH is a mild enhancer in itself and may play an important role in determining the interactions between the enhancers. Hence, contributions of the solvent cannot be decoupled from the observed potencies of enhancer mixtures. Change of solvent may have a major impact on enhancer potencies.

2.2. Screening for formulation potency

Full thickness porcine skin was used in all experiments. Skin was harvested from Yorkshire pigs and was stored at −70 °C immediately after procurement until the time of experiments. Formulation potency measurements were made using a high throughput screening tool, *IN vitro Skin Impedance Guided High Throughput screening* (INSIGHT), discussed in details elsewhere [38]. The INSIGHT screening apparatus consists of a teflon plate that serves as the donor and a polycarbonate plate that serves as the receiver, each 12.7 mm thick [39]. The donor contains a square matrix of 100 wells (each 3 mm in diameter) in the teflon plate that serve as individual donor compartments. A corresponding matrix of 100 wells in the polycarbonate plate serves as the receiver wells. The receiver wells were filled with PBS to keep the skin hydrated over the entire duration of the experiment (24 h). Skin was thawed at room temperature prior to each experiment. Thawed skin was placed between the donor and receiver plates with the stratum corneum facing the donor plate. The plates were clamped together using four screws. The skin was incubated with 85 μl of each test formulation in the donor wells for a period of 24 h with each formulation being repeated in four wells. Skin impedance in each well was recorded using two electrodes. One electrode was inserted into the dermis and served as a common electrode while the second electrode was placed sequentially into each donor compartment. An AC signal, 100 mV RMS at 100 Hz, was applied across the skin with a waveform generator (Agilent 33120A, Palo Alto, CA). Conductivity measurements were performed using a multimeter (Fluke 189, Everett, WA) with a resolution of 0.01 μA. Current measurements were performed at two time points, time 0 (I_0) and time 24 h (I_{24}). Enhancement ratio (ER) for each formulation was then calculated by taking the ratio of skin conductivities at 24 and 0 h. Synergy values, S, were calculated using the following equation.

$$S = \frac{\mathrm{ER}_{A+B}^{X,Y}}{X \cdot \mathrm{ER}_{A}^{Y} + (1-X) \cdot \mathrm{ER}_{B}^{Y}} \quad (1)$$

where $ER_{A+B}^{Y,X}$ is the enhancement ratio obtained with a formulation containing CPEs A and B at a concentration Y (0.5%, 1%, 1.5% and 2% w/v) and composition X ($0.1 \le X \le 0.9$) and ER_{A}^{Y} and ER_{B}^{Y} are the enhancement ratios obtained with pure CPEs A and B respectively at the same total concentration.

2.3. Franz diffusion cell studies

FDCs (16 mm diameter, 12 ml receiver volume) were used to assess the transport enhancements of test formulations. A small stir bar and a Ag/AgCl disk electrode (E242, InVivo Metric, Healdsburg, CA) were added to the receiver chamber. The receiver chamber was filled with PBS while taking adequate measures that no air was entrapped within the chamber. Thawed pig skin was mounted on the diffusion cell using a clamp with the stratum corneum side facing the donor. An AC signal, 100 mV RMS at 100 Hz, was applied across the skin with a waveform generator (Agilent 33120A, Palo Alto, CA). Conductivity measurements were performed using a multimeter (Fluke 189, Everett, WA) with a resolution of 0.01 μA. A radiolabeled tracer solute, inulin, was included in the formulation to be tested at a concentration of 10 μCi/ml. Labeled formulation was added to the donor compartment and held for a period of 48 h during which the receiver was sampled periodically. Concentration of radiolabeled inulin was measured using a scintillation counter (Packard Tri-Carb 2100 TR, Wellesley, MA). It was verified through an independent study that all detected radioactivity came from the model solute and not tritiated water that may have resulted from tritium exchange. Specifically, receiver samples were desiccated and analyzed for radioactivity and no significant difference was observed between native and desiccated receiver

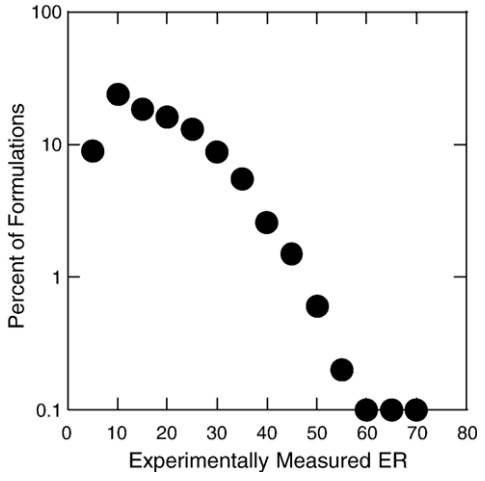

Fig. 1. Percentile distribution or likelihood of occurrence of ER values. A total of 4032 formulations resulting from 112 binary pairs were studied. Each pair was studied at 36 different permutations of compositions and concentrations. Plot was generated by binning ER data in ER bins of 10 units.

samples. Skin permeability was calculated using the standard equations. Control experiments were performed using PBS and 1:1 PBS/ethanol solutions.

3. Results

3.1. ER values

Formulations in the test library exhibited a wide range of ER values. Highest ER value of ~69 was observed for CBCAS: BDAC (2% w/v, CBCAS wt. fr. of 0.3). Blank formulation (1:1 PBS/EtOH) yielded an ER of ~3. The average value of ER for all formulations was 16.5±10.3 and the median value was 14.7. To put these numbers in perspective, the ER value of a formulation containing a commonly studied enhancer, oleic acid, at 1% w/v in 1:1 EtOH is 16.6. Distribution of ER is skewed in that it is densely populated at relatively low ER values (ER<20) and sparsely populated at high ER values, as can be clearly seen in Fig. 1. 51% or half of the formulations studied possessed ER<15, 97.4% formulations possessed ER<40 and 99.5% formulations possessed ER<50. The typical experimental errors in ER measurements are ~30%.

Fig. 2 shows a plot of experimentally measured ER values of binary formulations versus those predicted from a linear average of ER of their individual components. If binary formulations had exhibited pure additive effects, a good correlation between the two would be expected and the slope of the correlation would be equal to one. However, a poor fit was found between the two (r^2=0.32, slope=0.67). The data in Fig. 2 can be qualitatively clustered into three groups. A first group, the most populated, corresponds to formulations with good agreement between predicted and measured ER value (additive formulations). The second group shows measured ER significantly greater than the predicted ER (positively synergistic or protagonistic), and the third group shows formulations whose ER is significantly lower than the additive value (negatively synergistic or antagonistic). Quantitative classification of formulations into these groups depends on the choice of the threshold value (a good first approximation would be unity) which is used to distinguish synergistic formulations (both positive and negative) from additive ones. This threshold is arbitrary and is not discussed here. Nevertheless, the data in Fig. 2 show that a majority of formulations are additive in nature but many depart from this linear behavior.

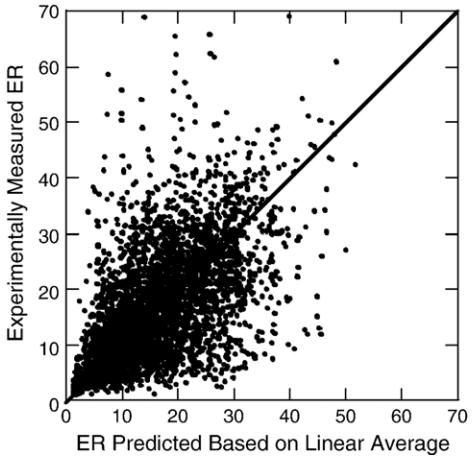

Fig. 2. Experimentally measured ER values of 4032 formulations plotted against predicted ER values based on linear averages of ER of participating individual components. A significant fraction of the data is centered around the line of identity.

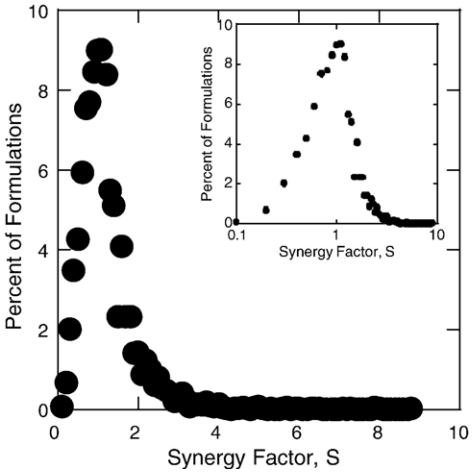

Fig. 3. Distribution of synergy values of all 4032 formulations studied here. Synergy data was binned in *S* bins of 0.1.

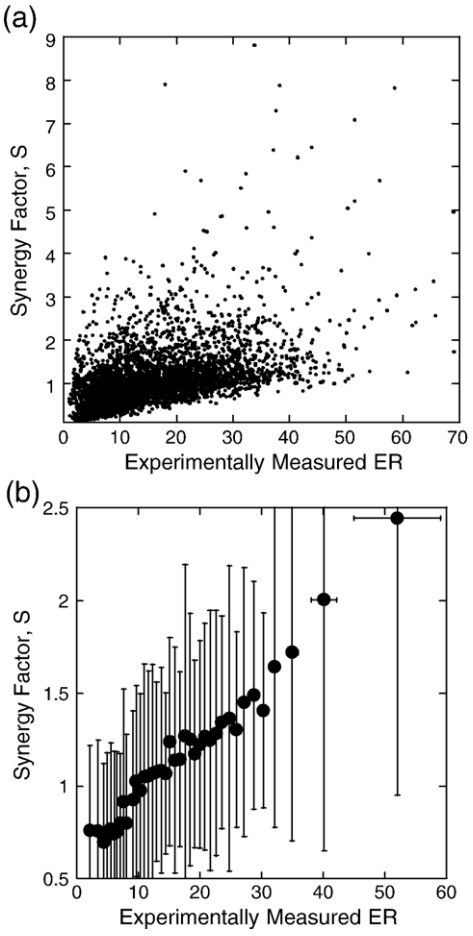

Fig. 4. (a) Correlation between ER and *S* for all formulations used in this study. (b) Correlation between ER and *S* averaged over 100 consecutive ER values from (a).

3.2. Synergy values

The conclusions in Fig. 2 can be seen in another light by calculating a synergy factor, S, for the formulations (as described by Eq. (1) in Methods). Briefly, S is a quantitative measure of the deviation of a formulation from its additive behavior. Thus, $S=1$ indicates a purely additive formulation, whereas $S>1$ or $S<1$ indicates a positively or negatively synergistic formulation respectively. Distribution of S values for all studied formulations can be seen in Fig. 3. A large cluster of data can be seen around $S\sim1$ confirming that a majority of formulations are indeed additive in nature. However, a significant number of formulations show moderately high values of S ($1<S<3$) and a small number of formulations show very high synergy values ($S>3$). A grand average of synergy values for all 4032 formulations is 1.1 ± 0.78 and the median value is 1.008. 69.7% formulations possessed S value between 0.5 and 1.5.

We also assessed the relationship between synergy and ER. Specifically, we probed if formulations with high ER usually correspond to highly synergistic formulations (Fig. 4a). No strong correlation was immediately apparent between ER and S at a population level ($r^2=0.2$, Fig. 4a). However, the lack of a correlation was mainly due to a large fraction of data clustered around $S=1$. This set shows significantly higher variability than the data on either extreme. The correlation between ER and S was evident when the data from either extreme was considered. Specifically, the average synergy factor of top 100 formulations (based on ER) was 2.25 ± 1.4 and the same number for bottom 100 formulations was 0.77 ± 0.47 and was statistically different (Student's t-test, $P<0.0001$). The correlation between ER and S over all formulations can be better seen by binning the data in groups of 100 (Fig. 4b). The correlation between the two was markedly improved ($r^2=0.97$).

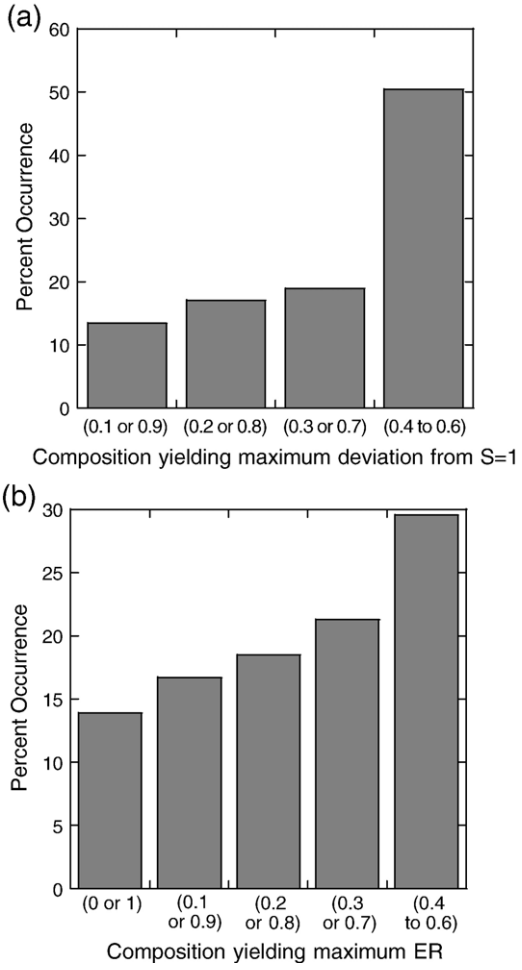

Fig. 5. (a) Composition-induced bias in the occurrence of the maximum synergy value in a given formulation (averaged over all 112 pairs). (b) Composition-induced bias in the occurrence of the maximum ER in a given formulation (averaged over all 112 pairs).

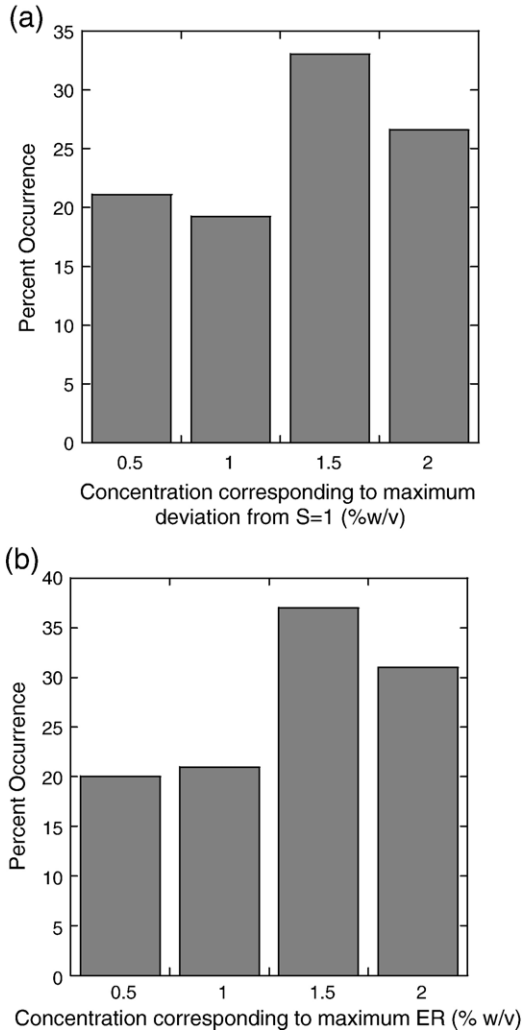

Fig. 6. (a) Concentration-induced bias in the occurrence of maximum synergy in a given formulation (averaged over all 112 pairs). (b) Concentration-induced bias in the occurrence of maximum ER in a given formulation (averaged over all 112 pairs).

3.3. Correlation of synergies with formulation composition

The low occurrence of high synergy values makes their identification challenging. Accordingly, knowledge of dependence of occurrence of synergies on formulation composition may be used to narrow the formulation space and increase the

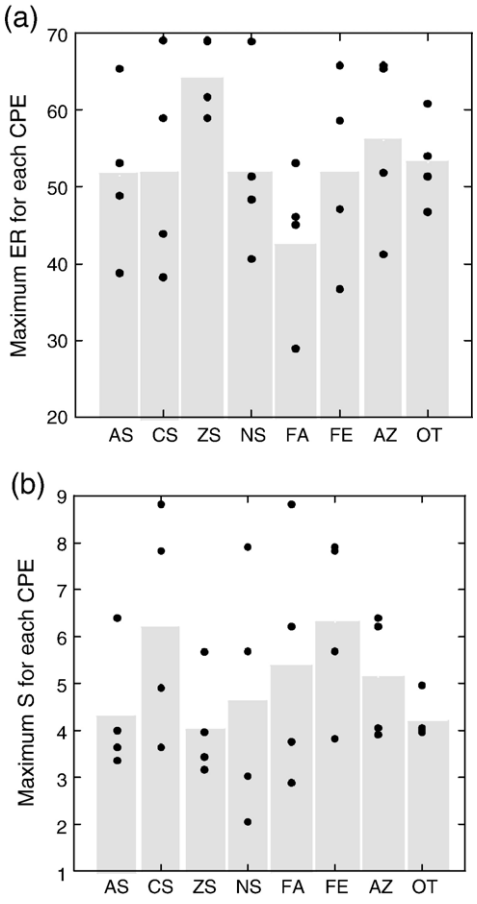

Fig. 7. (a) Distribution of maximum ER for CPEs in various groups (4 examples each). See Table 1 for abbreviations and list of CPEs in each category. (b) Distribution of maximum S for CPEs in various groups (4 examples each). See Table 1 for abbreviations and list of CPEs in each category.

efficiency of the discovery process. For each binary pair, we assessed all 36 formulations (44 formulations per binary pair of which 8 formulations contain single CPEs and 36 contain exactly two components) and determined the formulation which yielded highest deviation from $S=1$ on either side within that specific binary pair. Based on a similar analysis for all 112 binary pairs, we evaluated the likelihood of occurrence of maximum deviation at a specific concentration (0.5%, 1%, 1.5% or 2% w/v) or composition (0.1 to 0.9). The occurrence of maximum deviation showed a systematic dependence on composition. The occurrence was minimal near pure components (0.1 or 0.9, $P<0.01$, z-test, Fig. 5a) and increased dramatically at near-equimass compositions ($P<0.01$, z-test, Fig. 5a). A similar analysis was performed with respect to the composition and concentration at which maximum ER (ER_{max}) was observed for each binary pair. Occurrence of ER_{max} also exhibited a dependence on the composition. The likelihood of observing ER_{max} was minimal for pure components ($P<0.05$, z-test) and increased as the compositions approached near-equimass compositions; however, the maximum observed for the composition range of 0.4–0.6 was not statistically significant. The dependence of ER_{max} and maximum deviation of S on concentration was less pronounced. Fewer formulations exhibited ER_{max} and maximum deviation in S at lower concentrations (0.5% and 1% w/v, $P=0.06$, z-test) compared to higher concentrations (1.5% and 2% w/v, $P<0.05$ for 1.5% w/v, z-test) (Fig. 6a and b).

3.4. Dependence of S and ER on CPE chemistry

We probed whether there is a systematic dependence of S and ER on CPE chemistry. Values of ER and S, averaged over all formulations containing a specific CPE, showed no significant differences with respect to CPE category. This indicates that each CPE is capable of producing a range of ER and S values over various combinations. Next, we determined maximum ER and S for each CPE based on all formulations in which that particular CPE appears (each CPE participated in 252 formulations, 7 binary pairs and 36 formulations per pair). Fig. 7a shows maximum ER values for all CPEs, categorized by their chemistries (four CPEs per category, see Table 1). A wide range of maximum ER values was observed for all CPEs; however, some specific trends were apparent. Zwitterionic surfactants (ZS) produced significantly higher ER values compared to many other categories (t-test, $P<0.05$ compared to AS, FA and OT, $P<0.1$ compared to CS and

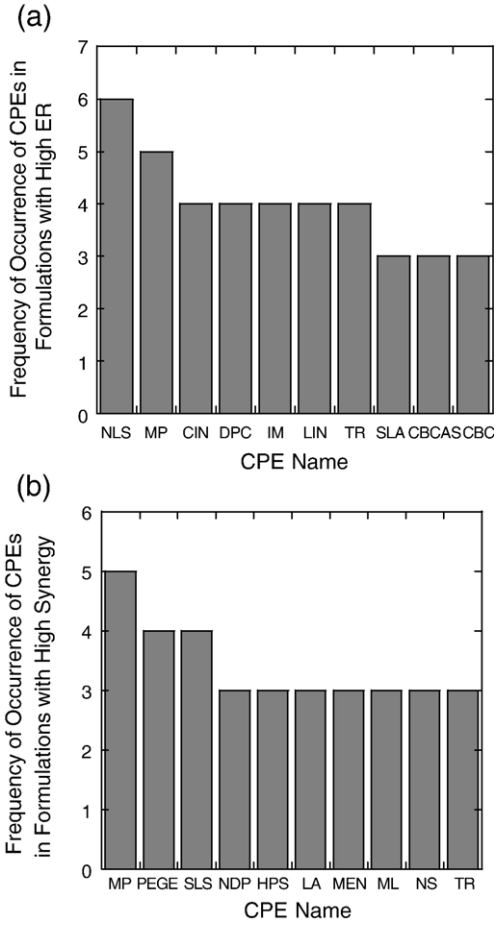

Fig. 8. (a) Occurrence of CPEs in top 32 pairs sorted based on maximum ER values. Only top 10 CPEs in the list are shown. Random occurrence of CPEs in this list corresponds to a frequency of 2. (b) Occurrence of CPEs in top 32 formulations sorted based on maximum S values. Only top 10 CPEs in the list are shown. Random occurrence of CPEs in this list corresponds to a frequency of 2.

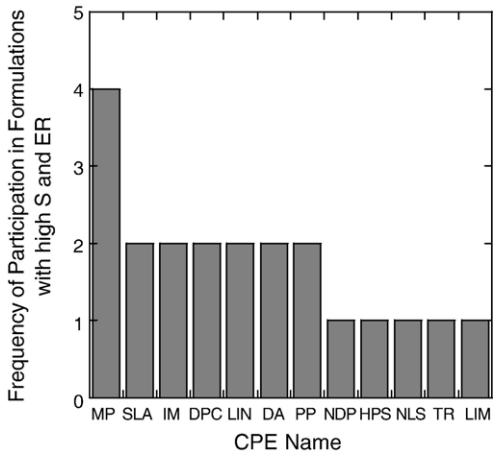

Fig. 9. CPEs present in top 32 pairs simultaneously picked on the basis of maximum ER and *S* values.

NS, and $P=0.12$ and 0.15 compared to FE and AZ, respectively). Similarly, fatty acids produced significantly lower ER compared to many other categories (*t*-test, $P<0.05$ compared to CS and ZS, $P<0.1$ compared to AS and OT, and $P>0.15$ compared to NA, FE and AZ). Maximum synergy values also showed variations with respect to CPE chemistry. Cationic surfactants showed high synergy values; however, the difference was not statistically significant in most cases (*t*-test, $P>0.1$ in all cases except compared to OT). Zwitterionic surfactants had lowest synergy values; however, the differences were not statistically significant in most cases (*t*-test, $P=0.052$ compared to FE, 0.13 compared to CS and >0.2 compared to all other cases).

Next we assessed whether certain CPEs are more likely to yield high ER and *S* values. We ranked all 112 binary pairs based on their ER_{max} values (maximum ER in a given binary pair). We then considered top 32 pairs and assessed how often do certain CPEs appear in this list. Ten CPEs that occur most frequently in this list are shown in Fig. 8a. Random occurrence of CPEs in this list corresponds to a frequency of 2. Several CPEs in Fig. 8a appeared at a frequency significantly greater than random occurrence. Two CPEs, MP and NLS, appeared most commonly. It is interesting that zwitterionic surfactants, which dominated the ER values in Fig. 7a, are not strongly represented in Fig. 8a. This owes largely to the fact that there was no particular zwitterionic surfactant that dominated their category and accordingly was not among the most frequent members of the list in Fig. 8a. Fig. 8b shows a similar plot of occurrence of CPEs in highly synergistic pairs. MP once again dominated the list.

The list of 32 pairs in Fig. 8a and b did not entirely overlap. There were 10 pairs common to both lists (for example, MP-DPC, IM-DPC, LIM-PP, SLA-PP and SLA-TR). Fig. 9 shows the frequency of appearance of CPEs in these common pairs. MP has a dominant presence in both lists. NLS, TR and SLA are among others that are also present in both lists.

4. Discussion

Analysis of a large number of formulations yielded interesting insights into the synergistic effects of CPEs on skin barrier properties. The results show, quite convincingly, that a random combination of two CPEs is not necessarily more effective than their individual components. A significant fraction of binary combinations exhibit additive rather than synergistic effects and the grand average of synergy values is close to unity. However, several binary formulations show significant deviations from the additive behavior. Such deviations become interesting in two contexts: positively synergistic formulations are very appealing for topical and transdermal drug delivery [38]. It is imperative to note, however, that a high positive synergy in ER does not necessarily imply improved safety. Previous studies have shown that safety of binary formulations can be substantially different compared to their individual components [38]. Secondly, formulations exhibiting negative synergies are relevant in the context of corrective or protective dermal formulations where the objective is to manipulate skin characteristics (such as moisture content, firmness, elasticity, etc.) with minimal effect on the barrier integrity.

The results presented here indicate that the likelihood of observing a formulation with certain ER is a strong function of ER itself. The probability of finding an ER value over 50 is less than 1%. This probability is likely to depend on the CPEs chosen in the library. CPEs in the current library were chosen to cover a wide range of chemistries without much emphasis on their potencies. Pre-screening of CPEs for potencies may significantly improve the success rate.

In light of the analysis presented here, we can now lay down a few general guidelines about selecting CPE libraries for future experiments. The results indicate that the likelihood of observing high synergies and ER is higher when both components are present at substantial concentrations. Based on these results, further libraries could emphasize formulations with compositions close to equi-mass or equi-molar ratios of both constituents. The results also indicated higher likelihood of occurrence of high synergies and ER values at higher concentrations. The concentration range used in this study (0–2% w/v) is somewhat arbitrary, although this was driven partly by the solubility issues for certain CPEs. However, for most CPEs used in the study, a concentration well above 2% w/v can be used. This needs to be explored in future studies. In addition, effects of solvents also require further considerations. Synergy values of CPEs and compositions corresponding to synergistic formulations are likely to shift upon change of the solvent.

The results also provide general guidance about selection of specific CPEs or classes of CPEs in future studies. As a group, zwitterionic surfactants provided higher values of ER_{max} compared to any other group; however, their synergy values were somewhat lower than those observed for other categories. At an individual CPE level, methyl pyrrolidone (MP) stood out over other chemicals. It was among the most frequently observed CPE in potent as well as synergistic formulations. These results are consistent with literature reports documenting synergistic effects of MP [40]. A few other CPEs, especially NLS and PEGE, also appear interesting.

The precise mechanisms by which synergies occur remains to be studied. Synergies may arise from the association between CPEs in the formulation thereby changing their interactions with

the skin. For example, cationic and anionic surfactants are highly likely to interact with each other and form complexes. Additional mechanisms also exist for CPE–CPE interactions especially through hydrogen bonding. CPEs could also possibly aggregate to form micelle-like structures. Since the presence of multiple surfactants is known to reduce the critical micelle concentration, it is possible that CPEs, which by themselves may not aggregate at concentrations used in this study, aggregate in presence of a second CPE. Alternatively, one CPE may simply increase partitioning of the other CPE in the skin in a non-linear manner and lead to synergistic effects. Mechanisms of negative synergies are also intriguing. Many CPEs, especially charge carrying molecules, are prone to precipitation upon addition of another CPE, especially of opposite charge. This could explain some of the negative synergy values. Alternatively, some of the CPEs could compensate for each other's effect. Many CPEs used in this study, especially surfactants, enhance skin permeability by lipid extraction, whereas others partition into skin [41]. Hence, one may theoretically be able to partly compensate the other's effect. Clearly, numerous possible mechanisms may explain synergistic behavior of CPE mixtures and detailed studies are necessary to understand them. In the absence of precise knowledge of mechanisms of synergistic interactions, information obtained from data mining provides a useful tool in improving screening efficiencies. It must be noted, however, that the rules presented here are applicable at an ensemble level. These rules are quite stochastic at an individual CPE level.

Combinations of CPEs provide an attractive modality for transdermal drug delivery. One of the major challenges in using chemical combinations, low success rate of formulation discovery, can be tackled by combination of knowledge-based discovery and high throughput screening. The leading hits from high throughput screening described in this study were assessed for their effect on skin permeability to inulin. Three best formulations (based on ER values) are listed in Table 2 (CBCAS-BDAC, NDP-TET and SLA-PP). Several other formulations representing various ranges of ER values (50s, 40s and 30s) are also shown. It is important to note that within a particular group, say ER between 40 and 50, potencies in terms of inulin permeability may not be statistically different. INSIGHT is a screening tool and is used to identify potent formulations from a large pool based on impedance measurement. The accuracy of INSIGHT predictions depends on the confidence of the relationship between skin permeability and impedance. This is discussed in detail elsewhere [42]. The ability of INSIGHT to distinguish two formulations depends on the difference in their impedance values. In other words, INSIGHT is accurate in identifying potent formulations (for example, ER > 40) from weak formulations (say ER ~ 10) but not necessarily in distinguishing formulations within the potent group (say ER = 50 and ER = 60). Inulin permeability from leading formulations was substantially higher than that from control values ($4–6 \times 10^{-4}$ cm/h compared to 7×10^{-6} cm/h). To put these values in perspective, tape-stripped porcine full thickness skin exhibited a permeability of about 7×10^{-4} cm/h. As another reference point, maximum permeability of porcine full thickness skin after application of lowfrequency ultrasound has been around 2×10^{-4} cm/h. It must be realized, however, that both physical methods (tape-stripping and ultrasound) permeabilize skin very quickly (in minutes) whereas chemicals require substantially longer time to act, between 24 and 48 h. On the flip side, chemical formulations are easily scalable to large areas whereas application of physical methods to large skin areas is challenging. Future studies need to be conducted to test extension of these studies to human skin.

Table 2
Inulin permeability values for selected formulations identified in this study

Binary pair	ER_{max}	Concentration (% w/v)	Composition (wt. fraction of first CPE)	Inulin permeability (cm/h) × 10^6
CBCAS-BDAC	69.1	2.0	0.3	305 ± 86
NDP-TET	65.8	2.0	0.4	446 ± 61
SLA-PP	65.4	0.5	0.7	563 ± 198
IM-DPC	58.6	1.5	0.3	465 ± 139
MP-DPC	54.0	1.5	0.4	579 ± 101
T20-MEN	51.4	2.0	0.5	612 ± 187
NLS-S20	48.3	1.0	0.6	239 ± 5
SLA-TR	45.1	2.0	0.2	170 ± 14
HPS-TET	44.5	1.5	0.3	374 ± 98
PEGE-BDAC	34.4	1.5	0.5	183 ± 23
SOS-LA	33.2	2.0	0.6	180 ± 29
CIN-LA	32.7	1.5	0.8	133 ± 9
Control (PBS)	2.0			7 ± 2
Control (1:1 PBS/EtOH)	2.9			10.7 ± 1.9

The first three formulations correspond to those with the three highest values of ER among 4032 formulations studied. The subsequent formulations correspond to examples from various tiers of ER values (50–60, 40–50 and 30–40, three examples each).

The results presented here clearly show that combinations of CPEs provide significant enhancements of skin permeability. Potencies of these formulations can be further improved by exploring higher concentrations, other solvents and using additional CPEs. While considering the utility of these formulations, the constraints imposed during their discovery must be kept in mind. Specifically, (i) no screening of safety is reported here. Selected formulations from high throughput screening can be tested for safety using *in vitro* or histological methods. Such data has already been reported for some of the formulations discussed here [38], (ii) the formulations discussed here were identified for long-term contact with skin. This constraint, however, can be easily overcome by modification of the screening protocol. For example, contact time can be reduced. Efforts are already underway to identify formulations that induce permeability changes after a short contact time (minutes), and finally (iii) many of the chemicals used in this study, especially surfactants, are not compatible with proteins due to their denaturizing properties. Accordingly, utility of these formulations for protein therapeutics should not be assumed. This constraint can be eliminated by pre-screening CPEs and solvent to limit the library to those which do not strongly interact with proteins.

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